Innate Sensing of miRNA Mimetics Provides Broad Range Antiviral Effects

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I dedicate this thesis to my parents and my beloved wife for their constant support and unconditional love.

"Real education enhances the dignity of a human being and increases his or her self-respect. If only the real sense of education could be realized by each individual and carried forward in every field of human activity, the world will be so much a better place to live in"

- Dr. A.P.J Abdul Kalam

1931-2015

Contents

Summary

Viruses are important disease agents in humans and present a worldwide health threat. Diverse structures and replication strategies of viruses elicit unprecedented complexities in the infected cells. This involves changes in signaling, gene expressions, metabolites and also lipids. Together these changes frequently drive the infected cell towards the state of a viral factory, where hundreds and thousands of progeny viruses are produced and eventually released. These features have been exploited for the development of antiviral drugs and vaccines. Synthetic short interfering RNAs (siRNA) and synthetic microRNAs (miRNA) are currently being developed and gaining prominence over the last few years (Wittrup & Lieberman 2015; Broderick & Zamore 2011). miRNAs are short ~22 nucleotide (nt) RNA molecules and bind to complementary sequences in the 3' UTR of multiple target mRNAs which can result in mRNA silencing. miRNAs are key modulators in several important cellular processes such as apoptosis, cell cycle control, and various physiological and developmental processes including haematopoiesis, stem cell differentiation, hypoxia, muscle development, insulin secretion, neurogenesis, aging, cholesterol metabolism, immune responses and viral replication (Bartel 2004).

In this study, we carried out the profiling of host microRNAs (miRNAs) in the course of human adenovirus (HAdV-C5 and HAdV-B3) infection of human epithelial lung A549 cells. The initial idea was to test the antiviral effect of differentially regulated miRNAs (miR-29b-1* and miR-27b*) at 24 h and 48 h post infection by overexpressing these miRNAs transiently through the transfection of commercially available mimics of miR29b-1* and miR-27b* on cultured cells. miRNA mimics are known to mimic or replicate the biological activity of the host miRNA. They are commercially synthesized double stranded (ds) RNA molecules with sequences identical to the host miRNA and represent a form of short non-self dsRNA. Such non-self RNAs can be recognized and sensed by host innate immune system through specific pattern recognition receptors (PRRs) (Uematsu & Akira 2008), such as toll-like receptors (TLR3) (Helen Flo & Aderem 2005), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) (Yoneyama et al. 2004) and melanoma differentiation-associated gene 5 (MDA5) (Yoneyama et al. 2005). Sensing and the activation of these sensors leads to antiviral response and the induction of type I interferon signaling in the host cell (Paz et al. 2006; Vasquez et al. 2005; Lazear et al. 2013; Panne et al. 2007). With this regard, we found that only blunt ended, non-modified miRNA mimics of miR29b-1* and miR27b* conferred broad-spectrum antiviral activity to the cells through the specific activation of RIG-I. Since the mature host miRNAs are known to have 2-nt overhangs at both the ends, these results clearly suggest that these mimics did not function as host miRNA. Instead, the antiviral activity of these mimics was mainly due to the sequence-specific activation of the dsRNA sensors mediated by the blunt ended structure of the mimics. We also studied the expression profiles of host genes which were differentially regulated by these mimics and obtained a list of potential targets. We focused on a subset of these targets to understand their mode of action in regulation the infection and found that the transfection of these mimics leads to NF-kB activation and IFN α/β secretion.

Zusammenfassung

Viren sind wichtige Krankheitserreger und stellen eine weltweite gesundheitliche Bedrohung dar. Verschiedene Strukturen und Replikationsstrategien der Viren rufen beispiellose Komplexitäten in infizierten Zellen hervor, darunter Veränderungen in der Signaltransduktion, der Genexpression sowie dem Stoffwechsel und Lipiden. Zusammen machen diese Veränderungen aus der infizierten Zelle eine virale Fabrik, in der Millionen von Viren produziert und freigesetzt werden. Diese Besonderheiten wurden für die Entwicklung von antiviralen Medikamenten und Impfstoffen ausgenutzt. Synthetische *short interfering RNAs* (siRNA) und *microRNAs* (miRNA) wurden in den letzten Jahren vermehrt zu diesem Zweck entwickelt (Wittrup & Lieberman 2015; Broderick & Zamore 2011). miRNAs sind kurze ca. 22 Nukleotide (nt) lange RNA Moleküle und binden an komplementäre Sequenzen im 3‘ UTR mehrerer mRNAs, was zum Gen-Silencing führen kann. miRNAs sind wesentliche Regulatoren in mehreren wichtigen zellulären Prozessen wie Apoptose und der Kontrolle des Zellzyklus, und in verschiedenen physiologischen und entwicklungsbiologischen Prozessen, darunter Hämatopoese, Stammzelldifferenzierung, Hypoxie, Muskelentwicklung, Insulinsekretion, Neurogenese, Altern, Cholesterinmetabolismus, Immunantworten und viraler Replikation (Bartel 2004).

In dieser Arbeit führten wir ein *Profiling* von zellulären miRNAs humaner Lungenepithel A549 Zellen im Verlauf einer Humanen Adenovirus (HAdV-C5 und HAdV-B3) Infektion durch. Ursprünglich sollte der antivirale Effekt differenziell regulierter miRNAs (miR-29b-1* and miR-27b*) nach 24 und 48 h post-Infektion untersucht werden, indem diese miRNAs transient mittels Transfektion von kommerziell verfügbaren *mimics* in kultivierten Zellen überexprimiert wurden. miRNA *mimics* replizieren die biologische Aktivität der zellulären miRNAs. Sie werden als doppelsträngige (ds) RNA-Moleküle mit identischer Sequenz synthetisiert und repräsentieren eine Art kurzer fremder dsRNA. Solche fremden RNAs können vom körpereigenen nativen Immunsystem durch Mustererkennungsrezeptoren (Uematsu & Akira 2008), wie z.B. Toll-like Rezeptoren (TLR3) (Helen Flo & Aderem 2005), *retinoic acid-inducible gene-i* (RIG-I) -artigen Rezeptoren (RLRs) (Yoneyama et al. 2004) und *melanoma differentiation-associated gene 5* (MDA5) (Yoneyama et al. 2005) detektiert werden. Die Aktivierung dieser Sensoren führt zu einer antiviralen Antwort und zu der Induktion der Typ I Interferon Signalkaskade in der Zelle (Paz et al. 2006; Vasquez et al. 2005; Lazear et al. 2013; Panne et al. 2007). In diesem Zusammenhang entdeckten wir, dass nur stumpf endende, nicht-modifizierte miRNA *mimics* von miR29b-1* und miR27b* eine breite antivirale Aktivität in der Zelle durch die spezifische Aktivierung von RIG-I hervorrufen. Da prozessierte zelluläre miRNAs 2-nt Überhänge an beiden Enden besitzen, deuten diese Ergebnisse darauf hin, dass diese *mimics* nicht als zelluläre miRNA wirkten. Stattdessen ist die antivirale Aktivität der *mimics* grösstenteils auf die sequenz-spezifische Aktivierung der dsRNA Sensoren über ihre stumpf endende Struktur zurückzuführen. Darüber hinaus analysierten wir die Expressionsprofile der zellulären Gene, die durch diese *mimics* differenziell reguliert wurden, und erhielten eine Liste möglicher Kandidaten. Die Untersuchung einer Untergruppe dieser auf ihre antivirale Wirkungsweise hin ergab, dass die Transfektion der *mimics* zur NF-kB Aktivierung und Sekretion von Interferon α/β führt.

Chapter 1

General Introduction

Introductory chapter of this thesis is divided into the three parts. **Part I** reviews the literature on general features of Adenoviruses such as virus structure, genome, life cycle and host cell responses to Adenovirus infection. **Part II** discusses host and viral miRNAs, their mode of action and biogenesis and their role in regulation the viral infection. **Part III** gives detailed introduction about the role of cytosolic DNA and RNA sensors in the host innate immunity and discusses about the viral strategies to block the sensor dependent innate immune activation.

Part I – Adenoviruses

1.1 General features of Adenoviruses

Adenoviruses were first isolated from human adenoids in 1953 (Rowe et al. 1953; Hilleman & Werner 1954). Several virus strains have been extensively studied in research and are used as tools to understand the mammalian cell and molecular biology. Adenoviruses are non-enveloped, icosahedral DNA viruses (70-90nm in size) (Figure 1.1) with a genome size of 26-45 kbp, capable of infecting many different vertebrates, including humans. Adenoviridae family consists of five genera: genus *Aviadenovirus*, genus *Mastadenovirus*, genus *Atadenovirus*, genus *Ichtadenovirus* and genus *Siadenovirus* (Rowe et al. 1953; Andrew M Q King et al. 2012). *Aviadenoviruses* only infect birds and are serologically distinct from other adenovirus genera. There are three viruses which belong to the genus *Siadenovirus*. One of them infects frogs (frog adenovirus) in particular and other two birds. *Atadenoviruses* are responsible for the infection of reptiles, marsupials, birds and ruminants and have distinct genomic organization and capsid. Recently discovered Ichtadenovirus has only one member known to infect fishes (white sturgeon adenovirus 1). Large genome size and phylogenetic differences differentiate it from other viruses (Andrew M Q King et al. 2012). Only the viruses belonging to the *Mastadenovirus* genus are capable of infecting mammals. There are more than 75 serotypes of human adenoviruses (documented in Genbank) and can be categorized into seven species (A-G) based on biochemical characteristics and sequence analysis (Benkö & Harrach 2003; Echavarría 2008). Viruses belonging to species B1, C, and E cause upper respiratory infections in children, and recurring infections in adults. Species F viruses are responsible for gastroenteritis. Some members belonging to D serotype cause keratoconjuctivites, while group B2 viruses are associated with kidney and urinary tract infections (Echavarría 2008). For immune-compromised individuals, adenoviruses are a major risk factor and virus infection can lead to severe health problems and fatalities (Kojaoghlanian et al. 2003). Species C human adenoviruses – HAdV-C2 and HAdV-C5 are the best studied adenoviruses, and these viruses are used as examples below to discuss the structure, life cycle and entry process of adenoviruses.

1.2 Adenovirus Structure

Figure 1.1 shows schematically the structure of HAdV-C2/HAdV-C5 particle (these two viruses are very similar, although they are distinct by serology). It has an icosahedral capsid (approx. 90nm diameter) composed of 240 hexon trimers. Each of 12 penton capsomers at the vertex contains a protruding trimeric fiber at the surface. Distal knob of the homotrimeric fiber functions as the attachment protein, whereas penton base proteins at the vertex of the capsid mediate virion internalization (Zubieta et al. 2005). Capsid is stabilized with the help of minor capsid proteins (IIIA, VI, VIII and IX) (Liu et al. 2010; Reddy et al. 2010). Linear double stranded DNA (~35

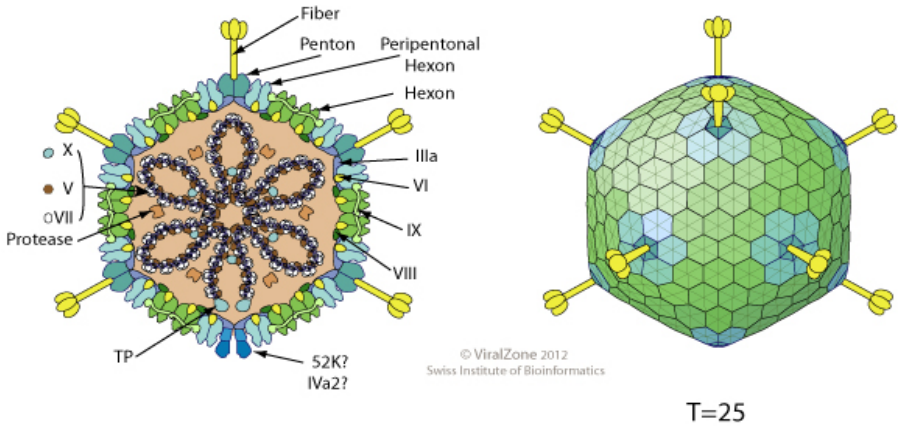

Figure 1.1: Structure of HAdV-C2 (http://viralzone.expasy.org)**.** The structure shown above is similar to all species of *Mastadenoviruses*. Shown is capsid shell (diameter = 90nm) consisting of 240 Hexon trimers and 12 vertex penton capsomeres. Fiber is shown protruding from the surface of each vertex.

kbp) is contained inside the capsid. Four DNA associated proteins (V, VII, X and terminal protein (TP)) form the virus core. Viral protease is additionally packaged into the particle.

1.3 Adenovirus life cycle

Life cycle of adenovirus depends on the permissivity for different strains of HAdV that varies greatly among the host cells. (**Table 1.1**). Productive multiplication of virus takes place in the permissive cells followed by lysis of the host cell. The steps involved in virus entry (**Figure 1.2**) and the overall life cycle of an adenovirus (**Figure 1.3)** is summarized in the section below.

1.3.1 Virus Entry

In most cases, virus entry into a susceptible host cell is mediated by the attachment of the fiber knob domain to the primary host cell receptor (Shayakhmetov et al. 2003). However, viral hexon is implicated in mediating binding to scavenger receptors. This is then followed by internalization of virus particles facilitated by the interaction of virus with a secondary receptor, which in the case of HAdV-C2/C5 is $\alpha v\beta 5$ or $\alpha v\beta 3$ integrin (Zhang & Bergelson 2005; Stewart & Nemerow 2007; Nemerow & Stewart 1999). The attachment receptor used for the virus entry depends on the particular species and serotype of the virus, for example, CAR is the attachment receptor for HAdV-A12, HAdV-C5, HAdV-C2, HAdV-E4 and HAdV-F41 (Roelvink et al. 1998) while CD46 and/or Desmoglein-2 (DSG-2) is mainly used by viruses belonging to subgroup B, that is CD46: HAdV-B35, HAdV-B16, HADV-B50 and HAdV-B2 (Tuve et al. 2006) and DSG2: HAdV-B7, HAdV-B3, HAdV-B14 and HAdV-B11 (Hongjie Wang et al. 2011; Sirena et al. 2004; Trinh et al. 2012). Other alternative attachment receptors implicated in HAdV entry include sialic acid, heparan sulfate glycosaminoglycans, CD 80/86, scavenger receptors and coagulation factors (Arnberg 2012; Zhang & Bergelson 2005; Wolfrum & Greber 2013). Attachment receptors used by HAdVs are shown in **Figure 1.4** and explained in detail in the section below.

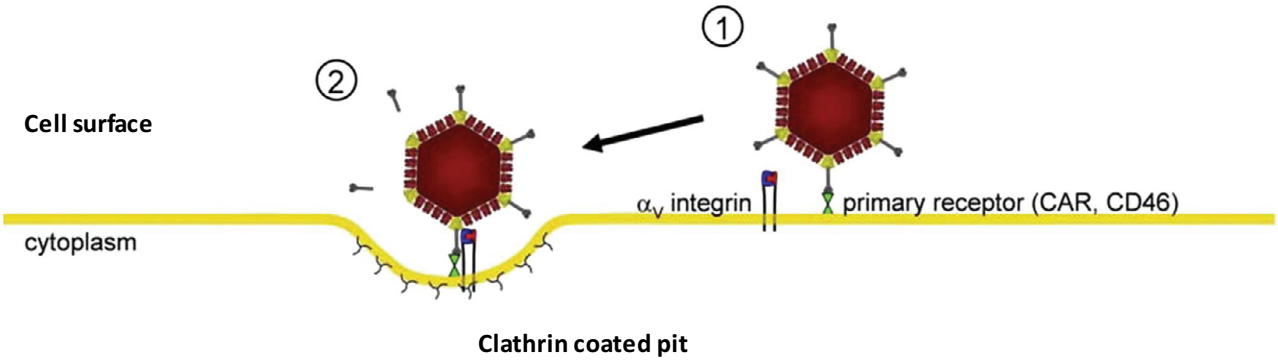

Figure 1.2: attachment and internalization of HAdV (Berk 2007). 1) HAdV binds to the primary receptor of the host cells such as CD46 (viruses belonging to species B) or CAR (Viruses belonging to species A, C, D, E, F) using viral Fiber knob. 2) HAdV penton base proteins interact with αV integrins through RGD sequences present in penton loops to promote endocytosis of the virus particle by causing the inclusion of the virus particle to clathrin coated vesicles. Subsequent steps in virus entry are discussed in **Figure 1.3.**

Subsequent to initial attachment, αv-integrins (αvβ5 & αvβ3) interact with the penton base and facilitate the internalization of virus particles by clathrin-dependent, receptor-mediated endocytosis of HAdV-C2/C5 (Meier & Greber 2003). On the contrary, HAdV-B3 and HAdV-B35 enter the cell by macropinocytosis (Amstutz et al. 2008; Kälin et al. 2010). Before Internalization, pulling forces generated on the fibers upon interaction with mobile CAR and stationary αv-integrins result in the fiber shedding and structural changes in the capsid which enable the externalization of membrane lytic protein VI (pVI) (Burckhardt et al. 2011). The entire process of HAdV attachment and internalization is shown in the (**Figure 1.2**). Disassembly of the virus continues in the endosome with the loss of protein IIIa and VIII (Greber et al. 1993). Endosomal membrane disruption and escape of the virus to the cytoplasm is promoted by membrane lytic protein VI (Burckhardt et al. 2011). This is followed by the dissociation of other viral proteins including penton base proteins (Greber et al. 1993). Soon after this disassembly, PPxy motif of pVI was shown to be essential for microtubule dependent trafficking of the virus towards the nucleus (Wodrich et al. 2010). However, the conclusions made in this study are debatable. Two other studies by (Kelkar et al. 2004) and (Bremner et al. 2009) suggested that hexon was important for cytoplasmic transport and that the transport was mediated by microtubule minus end-directed motor, dynein and its interaction with hexon. A similar conclusion, namely that hexon was the receptor for dynein motor complex was reached at by stochastic computationally modelling of experimentally determined microtubule dependent motion bursts measured on cytosolic virions (Gazzola et al. 2009). Before entering the nucleus, virus particles dock at the nuclear pore complex (NPC) by interacting with CAN/Nup214 (nuclear pore complex-filament protein) (Kelkar et al. 2004; Trotman et al. 2001). Protein IX (pIX) associated to viral hexon interacts with Klc1/2 (kinesin light chain) (Strunze et al. 2011). This is followed by Kif5C (kinesin-1 heavy chain) mediated disruption of the capsids via its interaction to Nup214 complex and microtubules. This disruption further leads to the dislocation of some nucleoporins and viral DNA is released into the nucleus (Strunze et al. 2011).

1.3.2 Virus replication and expression of late genes

Adenovirus replication requires the entry of viral DNA into the nucleus. Replication cycle of Adenoviruses can be divided into an early (E) and late (L) phase depending on the DNA replication process and the time of expression of specific viral genes. Host ribosome compatible numerous monocistronic mRNAs are generated by alternate splicing of primary transcripts. Early genes encode mainly non-structural, regulatory proteins. These proteins have three main functions: to regulate the expression of host proteins responsible for the DNA synthesis, to activate other viral genes (example, virus-encoded DNA polymerase) and to prevent the early death

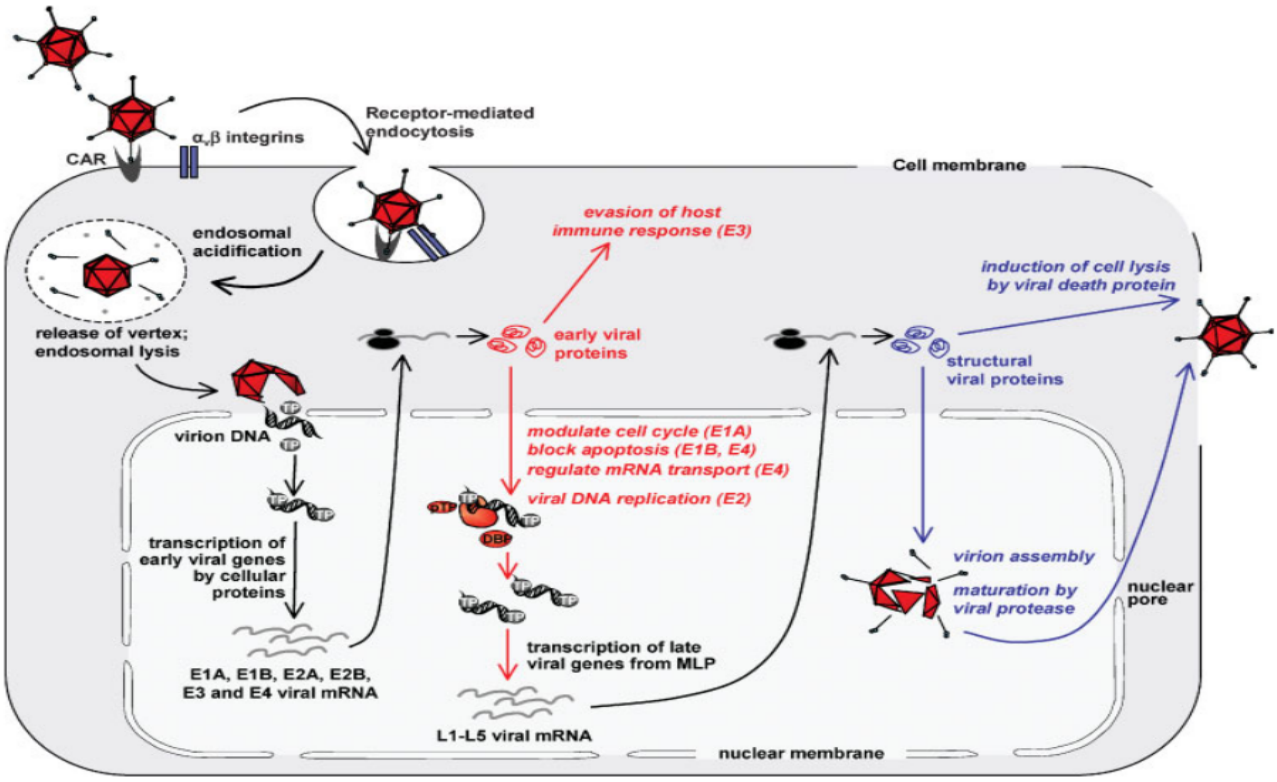

Figure 1.3: Overview of Human Adenovirus life cycle (Lenaerts et al. 2008)**:** Human Adenovirus (HAdV-C2) binds to CAR and is internalized by integrins via receptor mediated endocytosis. Virus escapes the endosomes and enters the nucleus by docking at the nuclear pore complex (NPCs). Microtubules are involved in the transport of virus through the cytoplasm to the nucleus and also in the final disassembly of the capsid at the NPC. Virus then releases its DNA into the nucleus. This is followed by the replication in the nucleus. Mature particles are formed and released upon the lysis of the infected cell which then initiate new round of infections.

of the infected cell by downregulating the host innate immune defences, such as apoptosis. E1A is the first viral gene to be transcribed. It provides favourable conditions for virus replication by promoting the S-phase of the cell cycle and by regulating antiviral innate immune response of the host towards infection (Russell 2000). E1A can activate transcription of other early genes such as E1B, E2, E3 and E4 (**Figure 1.3**) (Lenaerts et al. 2008;

Berk et al. 1979; Jones & Shenk 1979; Vijayalingam & Chinnadurai 2013). Accumulation of the E2 gene products (which include DNA polymerase, terminal protein precursor pTP and ssDNA binding protein) takes place at about 7h post infection (p.i.) (times may vary depending on the moi of virus used) (Fessler & Young 1998) thus provide optimal conditions for DNA synthesis. Synthesis of HAdV DNA is set to continue till the lysis of the host cells (Berk 2007). Additionally, cellular proteins also aid in the replication process (Mul et al. 1990). Replication is initiated when viral DNA polymerase interacts with primer pTP in a protein-priming replication mechanism. Such an interaction leads to the formation of preinitiation complex at the replication origin site present within the ITRs (inverted terminal repeats) of viral genome (Brenkman et al. 2002). Initiation of replication began opposite to the template strand (from the fourth base) upon covalent binding of dCTP (initiating nucleotide) to primer pTP at its serine residue (King & van der Vliet 1994; Lichy et al. 1981). Incorporation of the third nucleotide causes the jumping of the pTP-CAT (trinucleotide intermediate) to allow the base pairing to the template's first three nucleotides mediated by repeat sequences present in ITRs (King & van der Vliet 1994). Concomitantly, dissociation of viral polymerase and pTP starts to take place (King et al. 1997). Following this dissociation, further elongation is carried out by viral DNA polymerase and DBP. Once the duplication of one of the parental strands is complete, it gets displaced and can function as template in the form of single stranded DNA molecule for DNA synthesis (de Jong & van der Vliet 1999). Viral proteinase in the latter stages of infection, facilitates the cleavage of pTP (bound to ITRs) into smaller terminal proteins (Webster et al. 1989). Post replication, late genes (L1-L5) are expressed upon activation of MLP (HAdV major late promoter) (Nevins & Wilson 1981). Structural and virus assembly proteins, required for maturation and encapsidation of viral genomic DNA are encoded by 15-20mRNAs created by the splicing of a pre-mRNA transcribed by the late promoter. Structural proteins are transported from the cytoplasm to the nucleus for assembly. Trimerized hexon (major capsid protein) associates with minor capsid proteins and pentons in the nucleus. Packaging of the viral DNA and the assembly of the capsid is postulated to take place at the same time (Greber et al. 2013). However, if virion formation occurs by a concerted of a sequential assembly process is still a debated. Mutation studies on L1-52/55K and L4-33K proteins have suggested that these proteins along with special packaging sequence are responsible for packaging the Adenovirus genome (Gustin & Imperiale 1998; Finnen et al. 2001). After DNA encapsidation and capsid assembly, structural proteins are cleaved and processed into a mature form by the viral protease thereby making the virion infectious. The infected cells are finally lysed with the help of adenovirus death protein (E3-11.6K) of species C viruses and thereby release mature virus particles (Tollefson et al. 1996).

1.4 Classification, receptor usage and tropism

There are more than 70 serotypes of human Adenoviruses. They are categorized into seven species (A-G) based on genomic organization, DNA homologies, and hemagglutination properties. Based on receptor usage species B viruses can further be differentiated into two subspecies:B1 and B2. Ability of the host cell to support

the growth of a particular virus differs between adenovirus species and serotypes. Clinical diseases caused by HAdV largely depend on serotypes causing infection and include gastroentitis, conjunctivitis, pneumonia, myocarditis and hepatitis (table 1). Airway infections and disease pertaining to respiratory tract are caused by viruses belonging to species A-C and E (Chang et al. 2008). Viruses belonging to species B-E are mainly responsible for eye infections and conjunctivitis (Maranhào et al. 2009; Chang et al. 2008; Sambursky et al. 2007). Gastrointestinal tract infections are mainly associated with Species A, F, G and some viruses belonging to species B, C and D (Chang et al. 2008; A.M.Q. King et al. 2012). Species A and B are responsible for infections of urinary tract. Infections to the adenoids and tonsils are associated with species C adenoviruses. The cellular receptors used by HAdV to bind to the host cells is considered to be one of the key factors in determining the tropism. As mentioned above, binding of a virus to the primary receptor is vital for establishing a successful infection in the host (**Figure 1.4**). Coxsackie adenovirus receptor (CAR) is used as a primary receptor by viruses belonging to species A, C, D, E and F (Bergelson et al. 1997; Tomko et al. 1997; Roelvink et al. 1998; Lenman et al. 2015). In addition to CAR, HAdV belonging to species C and D are also known to use other molecules for attachment. These include heparin sulfate proteoglycans (HSPG) and scavenger receptor SR-A1 used by species C viruses (HAdV-C5) (Dechecchi et al. 2000) and sialic acid used by species D viruses (HAdV-D8, HAd-D19 and HAdV-D37) (Arnberg et al. 2000). SR-A1 present in Kupffer cells is used for HAdV-C5 uptake in the liver, if the virus is introduced into the host intravenously (Haisma et al. 2009). Uptake of HAdV-C5 in hepatocytes is mediated by initial binding of coagulation factor X to the virus (Dechecchi et al. 2000). This bridges the virus to the cell surface HSPG on hepatocytes and thus promotes virus entry into these cells (Waddington et al. 2008). CD46 is considered to be the primary receptor of most viruses belonging to species B (HAdV-B16, HAdV-B21, HAdV-B35 and HAdV-B50) (Gaggar et al. 2003; Sirena et al. 2004). Additionally, desmoglein 2 (DSG2) is used as receptor in some of species B viruses (HAdV-B3, HAdV-B7, HAdV-B11 and HAdV-B14), although Ad3 also binds to and uses CD46 as a receptor (Trinh et al. 2012; Sirena et al. 2004). HAdV-D26 which uses CD46 as its primary receptor (Li et al. 2012). Integrins act as secondary receptors and are used for the internalization of virus particles. So far 8β and 18α integrins have been found to be present in humans. The ones which promote the HAdV entry include $\alpha3\beta1$, $\alpha5\beta1$, $\alpha v\beta1$, $\alpha v\beta3$ and $\alpha v\beta5$ (Wickham et al. 1993; Li et al. 2001; Salone et al. 2003; Davison et al. 1997; Chiu et al. 1999). In the absence of additional attachment receptor, specific integrin could contribute to virus binding (Wickham et al. 1993).

1.5 Adenovirus genome

Human Adenovirus double-stranded DNA genome is non-segmented, linear and ~35kbp in size (Flint 1980; Roberts et al. 1984) (**Figure 1.5**). 5' end of the genome is covalently associated with terminal protein (primer pTP) (discussed above) (Webster et al. 1997; de Jong et al. 2003). mRNAs encoding majority of the adenoviral proteins are spliced (except for PIX and Iva2 units) and transcribed from the larger transcription unit (Biasiotto

& Akusjärvi 2015) by RNA polymerase II. Several differences exist between the genomes of adenoviruses belonging to different genera. Inverted terminal repeats are found at the ends of the genome, and as described above, these repeats have a central function in the viral DNA replication. The terminal repeats are followed by E1 and E4 transcription units at 5' and 3' ends, respectively, and these units exhibit large differences across genera. However, three proteins aiding the HAdV DNA replication, ssDNA binding protein, viral DNA polymerase and terminal protein-pTP (as described above) and major structural virion components are encoded by homologous genes found in all adenoviruses. Among human adenoviruses, HAdV-C2 genome was the first to be sequenced completely (Roberts et al. 1984). Several cis-acting viral packaging sequences were found to be repeated between the E1A region and the terminal repeat region on the left of the viral genome (Ostapchuk et al. 2005). These sequences are important for the generation of infectious particles by facilitating efficient packaging of HAdV DNA.

Table 1: Clinical diseases caused by different HAdV serotypes (Jones et al. 2007; Madisch et al. 2007; Aoki et al. 2008; Walls et al. 2003; Feigin 2014; Echavarría 2008; Erdman et al. 2002; Meyer-Rüsenberg et al. 2011)

HAvD subgroup	Serotype	Type of infection
A	12, 18, 31	gastrointestinal, respiratory, urinary
B, type 1	**3, 7, 16, 21**	keratoconjunctivitis, gastrointestinal, respiratory, urinary
B, type 2	11, 14, 34, 35	gastrointestinal, respiratory, urinary
C	1, 2, 5, 6	respiratory, gastrointestinal including hepatitis, urinary
D	**8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49**	keratoconjunctivitis, gastrointestinal
E	**4**	keratoconjunctivitis, respiratory
F	40, 41	gastrointestinal
G	52	gastrointestinal

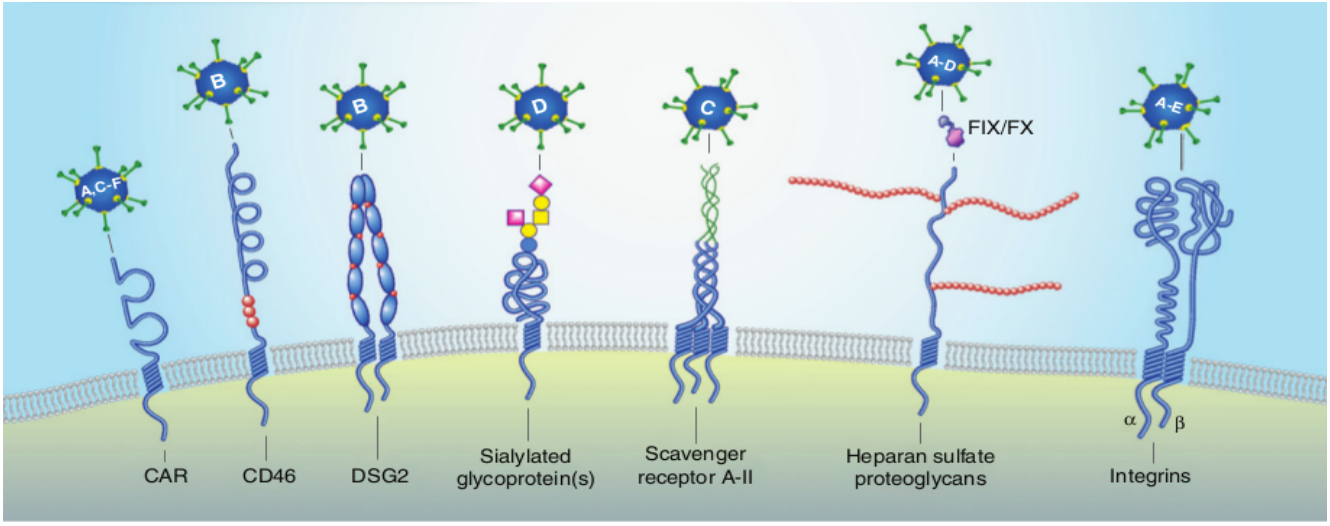

Figure 1.4: Cell surface receptors used by HAdV (Arnberg 2012)**.** Virus belonging to species A, C-F use CAR for cellular attachment. CD46 and/or DSG2 is used mostly by B species. Other receptor shown here are used by viruses belonging to single species. Integrins promotes virus internalization.

E1A, E1B, E2, E3 and E4 represent five early transcription units encoded by HAdV genome, E1A being the first unit transcribed. Transcription of four intermediate units (Iva2, IX, E2 late and L4 intermediate) follows and transcription of one major late unit commences after HAdV DNA replication (Törmänen et al. 2006). Human and primate adenoviruses belonging to Mastadenoviruses are known to encode also one or two virus-associated VA-RNAs (VA-RNAs). VA-RNAs transcribed by RNA polymerase III are known to block parts of host miRNA machinery (Davison et al. 2003). They are produced in high amounts and compete with host miRNAs for the nuclear export factor exportin.

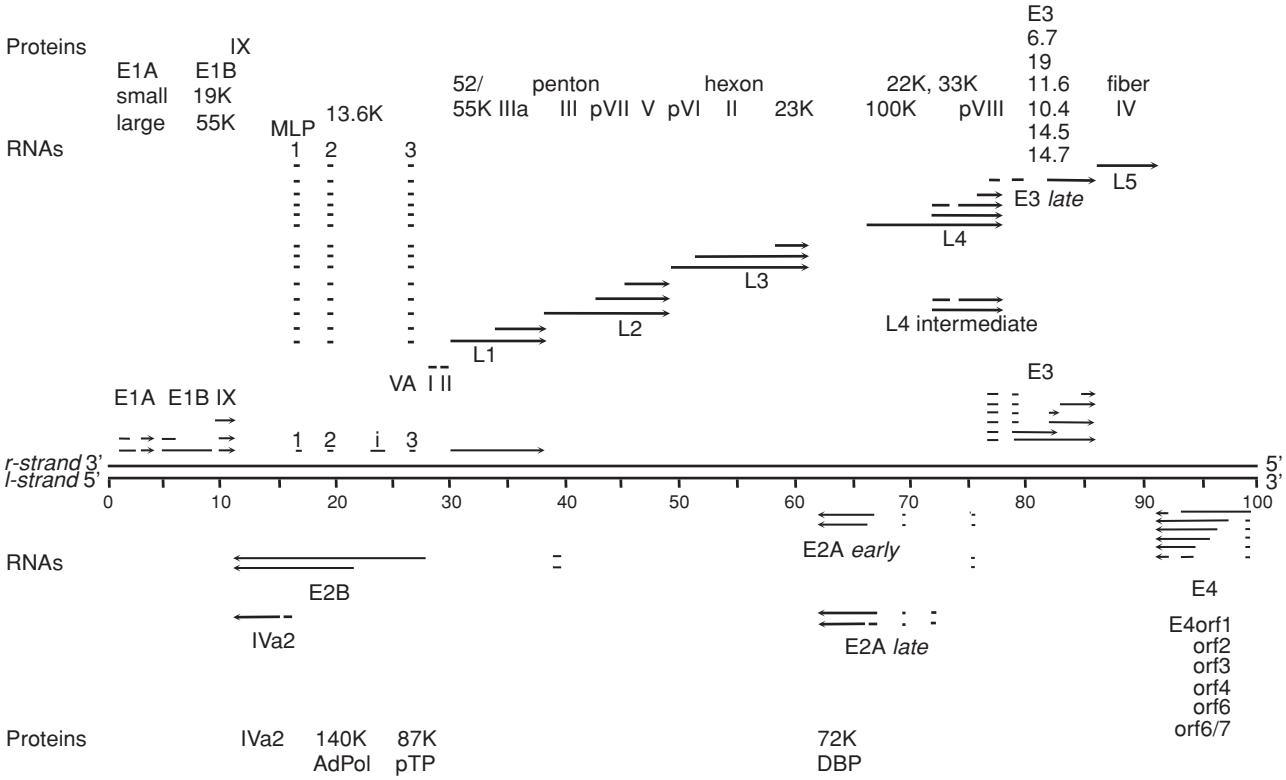

Figure 1.5: Overview of HAdV-C2 and HAdV-C5 genome (Berk 2007). Shown here is the 100 map units of HAdV-C2 genome (35937 bp). Thin lines represent the early mRNAs and their exons, heavy lines denote late mRNAs and lines with intermediate thickness represent intermediate RNAs (Iva2, IX, E2Alate and L4 intermediate). Polyadenylated 3' ends are denoted by arrowheads. Major late promoter is present at 16.8 map units and is responsible transcription of late mRNAs. Exons (labelled as 1,2 and 3 in the figure) of the tripartite leader sequence are found at the 5' of all late mRNAs. E2 region mediates the expression of E2B mRNAs which encodes for pTP and HAdV DNA polymerase. Proteins named at the top represent the ones which are translated from mRNAs transcribed rightwards and the ones named at the bottom denote the proteins translated from the leftwards transcribed mRNAs, Viral protein III (pIII) is also referred as the penton base. Viral protein II (pII) represent the hexon and fiber is designated as protein IV (pIV). Upon maturation, precursor polypeptides (pVII, pVI, pVIII) are cleaved.

Finally, I would like to briefly point out the important functions associated to the proteins or polypeptides encoded by the HAdV transcription units. Detailed discussion of functions of each viral protein is beyond the scope of this chapter. As described above, two proteins encoded by the E1A transcription unit (289R and 243R) are required to initiate the transcription of other viral genes and trigger the cells into S-phase of the cell

cycle (Bayley & Mymryk 1994; Flint & Shenk 1997). Two proteins are encoded by E1B (E1B-19K and E1B55K). E1B-19K is involved in the inhibition of apoptosis of the infected cells (Chiou et al. 1994; White 2006). E1B-55K in complex with E4orf6 and other cellular proteins (elongins B and C, RING-box-1 and cullin 5) promotes the formation of E3 ubiquitin ligase complex. P53 (known cellular target of this ubiquitin ligase) as a result is subjected to proteasomal degradation (Querido et al. 2001). This inhibits cellular DNA damage response, promotes the transport of viral mRNA from the nucleus and blocks cellular mRNA export (Woo & Berk 2007; Blanchette et al. 2008). E3 region is dispensable for virus replication in cell culture, but in-vivo, proteins encoded in this region modulate host immune response to the infection (Fessler et al. 2004; Lichtenstein et al. 2004). Family of late mRNAs are associated with capsid production and its assembly. The products encoded by E4 are associated with the wide range of functions such as functions such as RNA splicing (Bridge et al. 2003), nuclear export of viral mRNAs (Weigel & Dobbelstein 2000), However one main function of E4 proteins (E4orf3 and E4orf6+E1B55K complex) is to suppress DNA damage response (Weitzman & Ornelles n.d.).

1.6 Impact of adenovirus infection and host cell response

Virus infection can be sensed by host innate immune system in a number of ways as soon as virus gains entry into the host cell. Such sensing and the prompt activation of antiviral factors are important for the survival of the infected host. Hosts have developed multiple strategies over a period of time to recognize non-self genetic material from pathogenic determinants such as viruses. HAdV infection induces the host immune system and activates multiple antiviral defence mechanisms. Several studies have reported the activation of type-I interferon pathway, secretion of pro-inflammatory cytokines and activation of inflammasomes leading to the antiviral response during the early stages of HAdV infection by antigen presenting cells (APCs) such as dendritic cells and macrophages (Zhu et al. 2007; Nociari et al. 2007; Lam et al. 2014). These cells can sense viral DNA, capsid proteins and virus-associated VA-RNAs (**Figure 1.6**) (Nociari et al. 2007; Muruve et al. 2008; Yamaguchi et al. 2010; Lam et al. 2014). Type 1 interferons (IFNα and IFNβ) released by the HAdV-infected cells positively regulate the expression of several antiviral effector genes thereby alerting the non-infected cells neighbouring the infected cells of a possible viral infection and inducing antiviral state in these cells (Randall & Goodbourn 2008). Considering the complex nature of the immune response towards HAdV infections, the detailed explanation of different signalling pathways and specific roles of the factors involved in viral clearance from the host is beyond the scope of this introduction. Based on the data available from recent studies, I would like to briefly review the mechanisms involved in host sensing of HAdV infections.

Several studies have demonstrated cell-specific immune response against adenoviruses, particularly in the experiments involving APCs derived from bone marrow (Fejer et al. 2008; Nociari et al. 2009; Nociari et al. 2007; Zhu et al. 2007). In these studies, induction of type 1 Interferon pathway was the main feature of antiviral response. IRF3 (interferon response factor 3) activation was observed and marked the early induction

of antiviral response. Upon endosomal virus uptake by the cells, phosphorylation of IRF3 took place without the activation of TLRs (Toll-like receptors). This activation was facilitated by viral DNA being sensed in cytosol following its escape from the endosomes (Figure 1.6) (Nociari et al. 2007). Studies performed in murine cells responsive to adenovirus type 5 vectors deficient in replication (rAdV) reported that the phosphorylation of IRF3 required the STING/TBK1 module (Stein & Falck-Pedersen 2012; Stein et al. 2012; Ishikawa & Barber 2008; Ishikawa et al. 2009). STING operates as an adapter to cause the activation of TBK1 kinase and thereby links the DNA sensing to signaling (Ishikawa & Barber 2008; Ishikawa et al. 2009). Phosphorylated IRF3 dimerizes and enters the nucleus (Servant et al. 2003; Clément et al. 2008).

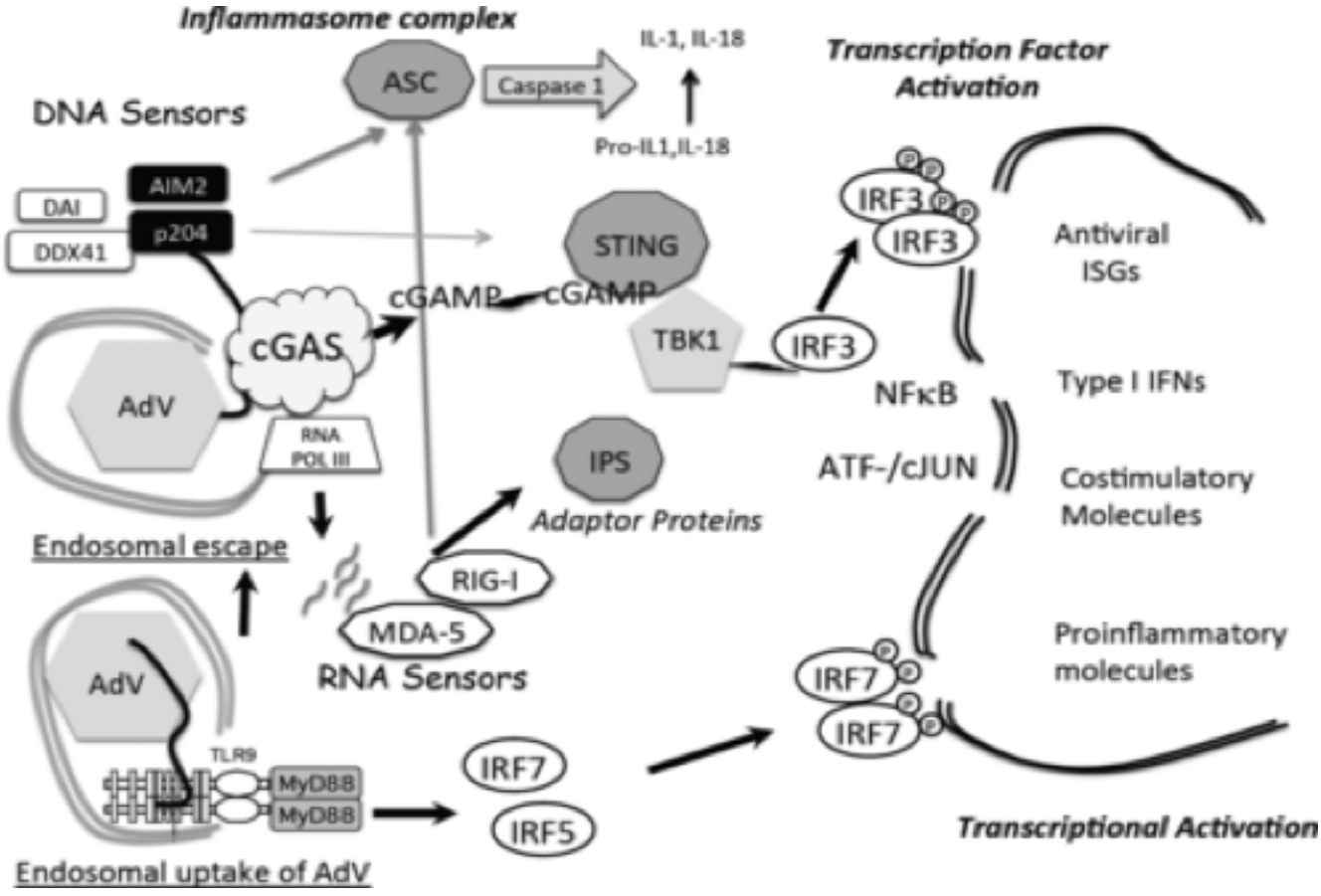

Figure 1.6: Adenovirus sensing in Macrophage like cells (RAW264.7) (Lam et al. 2014). Upon endosomal uptake of the virus, TLR9 expressed in endosomes senses adenoviral DNA to activate TLR9 pathway. This causes the activation of IRF5 and IRF7 in a MyD88 dependent mechanism. Additionally, upon endosomal escape, viral DNA becomes accessible to the wide range of cytosolic DNA sensors. One of them, cGAS (cyclic GMP-AMP synthase) acts as a primary sensor in recognizing adenovirus infection and promotes antiviral response in murine macrophage and endothelial cell lines. cGAMP produced as a result of this sensing activates STING, which in turn leads to activation of TBK1. IRF3 is phosphorylated by TBK1, and phosphorylated IRF3 dimerizes and translocates to the nucleus (shown in the figure as a double line structure). Here it interacts with other transcription factors (ATF/cJUN and NF-kβ) to induce the expression of ISGs. STING-TBK1-IRF3 axis leads to activation of type 1 interferon transcription and activation of inflammasomes leads to caspase 1 activity. Viral RNAs (VA-RNAs) transcribed from nuclear adenovirus genomes contain double-strand RNA structures and can be sensed by RIG-I.

Inside the nucleus, IRF3 activates the IFN-β gene and secretion of IFN--β (Panne et al. 2007), thereby mounting antiviral response against virus infection (**Figure 1.6**). A study in respiratory tract epithelial cells demonstrated that binding of the primary receptor CAR to the fiber triggers an early immune response by causing the induction of downstream signalling of MAPK, JNK and ERK1/2 during infection. This event leads to the activation of pro-inflammatory genes and NF-kB (Tamanini et al. 2006). This event leads to the activation of pro-inflammatory genes and NF-kB. Upon virus internalization, viral DNA exposed in the endosomes is sensed by toll-life receptor 9 (TLR9). This sensing results in the activation of Interferon regulatory factors IRF5 and IRF7 (constitutively expressed only in the dendritic cells) (Stein & Falck-Pedersen 2012; Barlan et al. 2011). TLR9 is abundantly expressed only in plasmacytoid dendritic cells (pDCs). Hence the role of TLR9 as primary sensor for infection seems to be relevant only in these cells (Zhu et al. 2007). In summary, the antiviral response exhibited by different cell types occurs by the combined effect of primary and secondary responses discussed above.

1.6.1 Role of DNA sensors

A large number of DNA sensors (AIM2, DAI, RNA pol III, DDx41 and IFI16)) have been extensively studied in recent times (Holm et al. 2013). The findings on STING activation (Burdette et al. 2011; Sauer et al. 2011; Huang et al. 2012; Prantner et al. 2012; Shu et al. 2012) by cGAS (GMP-AMP synthase) acting as a DNA sensor (Sun et al. 2013; J. Wu et al. 2013) suggested the association between DNA sensing and the downstream antiviral signaling. Lam et al. 2014 reported that activated cGAS led to the production of cGAMP (cyclic guanine-adenine dinucleotide) (Ablasser, Goldeck, et al. 2013; P. Gao et al. 2013; Zhang et al. 2013; Diner et al. 2013) and caused the activation of STING (**Figure 1.6**). The evidence that cGAS binds to adenovirus DNA and could potentially function as the primary cytosolic sensor for the viral DNA was later provided by Lam et al. 2014. Adenoviruses can also activate other sensors including some of RNA sensors in the cytoplasm. VA-RNA encoded by human adenoviruses can be sensed by RIG-I leading to the induction of type I IFN response (Minamitani et al. 2011).

1.6.2 Immune evasion strategies

To overcome these cellular responses and escape detection and sensing by immune system, virus has evolved counter mechanisms. Viral proteins such as E1A, E1B and E3 effectively block immune response pathways by interacting with various regulators of the immune system. E1A is responsible for blocking ISGF3 complex (consisting of IRF9 and STAT1-STAT2 heterodimer). ISGF3 is activated upon induction of type 1 Interferon signalling and during enhanced expression of ISGs and IRFs. Upon activation, ISGF3 is translocated to the nucleus. This complex inside the nucleus interacts with specific nucleotide sequences known as ISREs (IFN-stimulated response elements) located in the promoter region of the ISGs. This results in the induction of ISGs

(Kessler et al. 1990; Veals et al. 1993). Monoubiquitination of H2B (Histone H2B) by hBre1 complex (hBre1, RNF-40 and Ube2b) at the promoter and the transcribed region of ISGs is essential for the antiviral activity mediated by IFN. It was found that E1A repressed ISG expression by disrupting hBre1's interaction with Ube2b which was required for H2B ubiquitination (Fonseca et al. 2012). Blocking apoptosis of an infected cells is essential for completing the virus life cycle and formation of new infectious particles. E1B-19K prevents the premature apoptosis of the infected cell (Chiou et al. 1994; White 2006). In HAdV-C5, E1B-55K protein also plays an important role in negatively regulating type-I interferon response of cultured HEK293 cells, but the exact molecular mechanism is still unclear (Chahal et al. 2013). E3 region encodes for proteins which inhibit the transcription induced by NF-kB (E3-14.7K), TNFα and cell death induced by FAS ligand (E3-10kDa/14.5kDa) and peptide presentation by class I MHC (E3-19k) (Thaci et al. 2011).

Part II – miRNAs

Control of mRNA degradation and translation are major processes in the regulation of gene expression in the host (Shyu et al. 2008). A tight multistage control of gene expression is essential for the normal functioning of the cells and is regulated at multiple levels by host micro RNA (miRNA) (He & Hannon 2004; Jackson & Standart 2007; Shivdasani 2006). miRNAs are short (19-25 nucleotides long) non-coding RNAs which can post transcriptionally regulate the expression of large number of target genes by binding mostly to the 3' untranslated regions (UTRs) and in some cases to the 5'UTR or the coding regions of target messenger RNAs (mRNAs) (He & Hannon 2004; Filipowicz et al. 2008; Ambros 2004; Bartel 2004). lin-4, the first miRNA discovered in C. *elegans* was shown to be involved in post-embryonic development (Lee et al. 1993). Over the past decade, miRNAs and their regulation of host genes have been extensively studied by several research groups. miRNAs are involved in the regulation of important cellular processes such as cell division and differentiation, apoptosis and cell cycle control, and various physiological and developmental processes including haematopoiesis, stem cell differentiation, hypoxia, muscle development, insulin secretion, neurogenesis, aging, cholesterol metabolism, immune responses and viral replication (Bartel 2004; Miska 2005; Zamore & Haley 2005; Kloosterman & Plasterk 2006). In June, 2014, 35828 mature miRNAs of 223 species were annotated in global miRNA collection database (miRBase) (http://www.mirbase.org). This database has a collection of 502 mature viral miRNAs and 1181 mature human miRNA sequences. The importance of these small RNAs in regulating the large number of genes simultaneously can be attributed to highly conserved nature of their target binding sites and the presence of large number of these molecules in an organism (Kozomara et al. 2014; MacFarlane & Murphy 2010). Experimental and computational analyses have revealed that more than 60% of genes coding for proteins undergo miRNA regulation (Friedman et al. 2009; Lewis et al. 2005).

2.1 miRNA nomenclature

A commonly accepted system for the nomenclature and annotation has been developed for cataloguing miRNA (Ambros et al. 2003; Griffiths-Jones 2004; Griffiths-Jones et al. 2006; Griffiths-Jones et al. 2008). miRNAs are sequentially numbered in the order of their discovery. Once confirmed experimentally, a number is assigned to a miRNA with a prefix 'miR' followed by '-' (e.g. miR-29b). Each miRNA is given an identifier which includes the first three letters of the organism to which it belongs (bta for *Bos taurus*, hsa- for *Homo sapiens*). Mature miRNA can be characterized with the capitalized 'R' letter in the name 'hsa-mi**R**-29b' while 'mir-29b' represents miRNA gene or precursor miRNA with stem loop structure. Distinct precursor sequences and genomic loci that express identical mature sequences get names of the form hsa-mir-29b-1 and hsa-mir-29b-2. Mature miRNA sequences which are closely related to each other and which differ by 1 or 2 nucleotides

contain a lettered suffix in their names. Three known precursors of miRNA-29 family include hsa-miR-29a, hsa-miR-29b and hsa-miR-29c (Kriegel et al. 2012). Studies from deep sequencing have shown that single pre-miRNA can produce many mature miRNA molecules with variable lengths and sequences called 'isomirs'. This variation is the result of modifications which includes insertions, substitutions, deletions or trimming of 5' or 3' ends. Those miRNAs which are abundantly expressed are named in the same manner as discussed above (e.g. hsa-miR-21) while the strand opposite to this precursor is given the same name but with an asterisk following the number (has-miR-21*). However, If the expression levels of either strand is not possible to be determined, identifiers like miR-146b-5p (strand from the 5' end) or miR-146b-3p (strand from the 3'end) are assigned. miRNAs can sometimes be present as clusters and found in close proximity to each other in the genome. For example, 6 pre-miRNAs present within 1kb region of chromosome 13 form miR-17 cluster. Maturation of these precursor forms yield 7 mature miRNAs (miR-17, miR-18, miR-91, miR19, miR-92, miR-20 and miR-19b) (Lee et al. 2002). In the literature, this cluster is also referred to as miR-17 or miR-17-92 cluster.

2.2 miRNA biogenesis

Figure 2.1 shows the kay steps involved in the biosynthesis of miRNAs. miRNAs are transcribed as primary miRNAs (pri-miRNAs) by RNA polymerase II or RNA polymerase III (Lee et al. 2004; Borchert et al. 2006). These long primary transcripts are encoded in intragenic (both intronic and exonic) or intergenic, regions of the genome and contain 5' cap and poly-adenylated 3'end known as pri-miRNAs (Rodriguez et al. 2004). It is estimated that about 50% of the non-protein coding transcripts encode for miRNAs (Saini et al. 2007). These pri-miRNAs are folded and contain multiple hairpin loops, and they are processed and cleaved into 70-120 nucleotide long precursor-miRNA (pre-miRNA) by the cellular classII RNaseIII Drosha – DGCR8/Pasha protein complex also referred to as microprocessor (**Figure 2.1A**) (Lee et al. 2003; Denli et al. 2004; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004) (Lee et al. 2003; Denli et al. 2004; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004). This pre-miRNA contains a 2-nucleotide overhang at the 3' end and a phosphate group at the 5' end.

The RAN-GTP dependent, nuclear transport receptor protein - Exportin-5 is responsible for the export of newly transcribed pre-miRNA from nucleus to cytoplasm (**Figure 2.1B**) (Lund et al. 2004; Yi et al. 2003). Here, pre-miRNA is further cleaved and processed into a mature 19-24bp long double stranded miRNA with imperfect complementarity by DICER-I (RNAse III endonuclease enzyme) and RNA binding (TRBP)/PACT proteins (Yi et al. 2003; Chendrimada et al. 2005; Lee et al. 2006; Lee et al. 2002) (**Figure 2.1C, D**). These double stranded RNA molecules with overhangs and imperfect complementarity are unwound with the help of helicase into a 22-24 nucleotide long mature miRNA. The separation of the miRNA strands depends on the base pairing stability at 5' end and the thermodynamic asymmetry of the miRNA duplex (Bhaskaran & Mohan 2013). One of the strands of the miRNA duplex, the guide strand in complex with DICER-1 and TRBP/PACT protein associates with

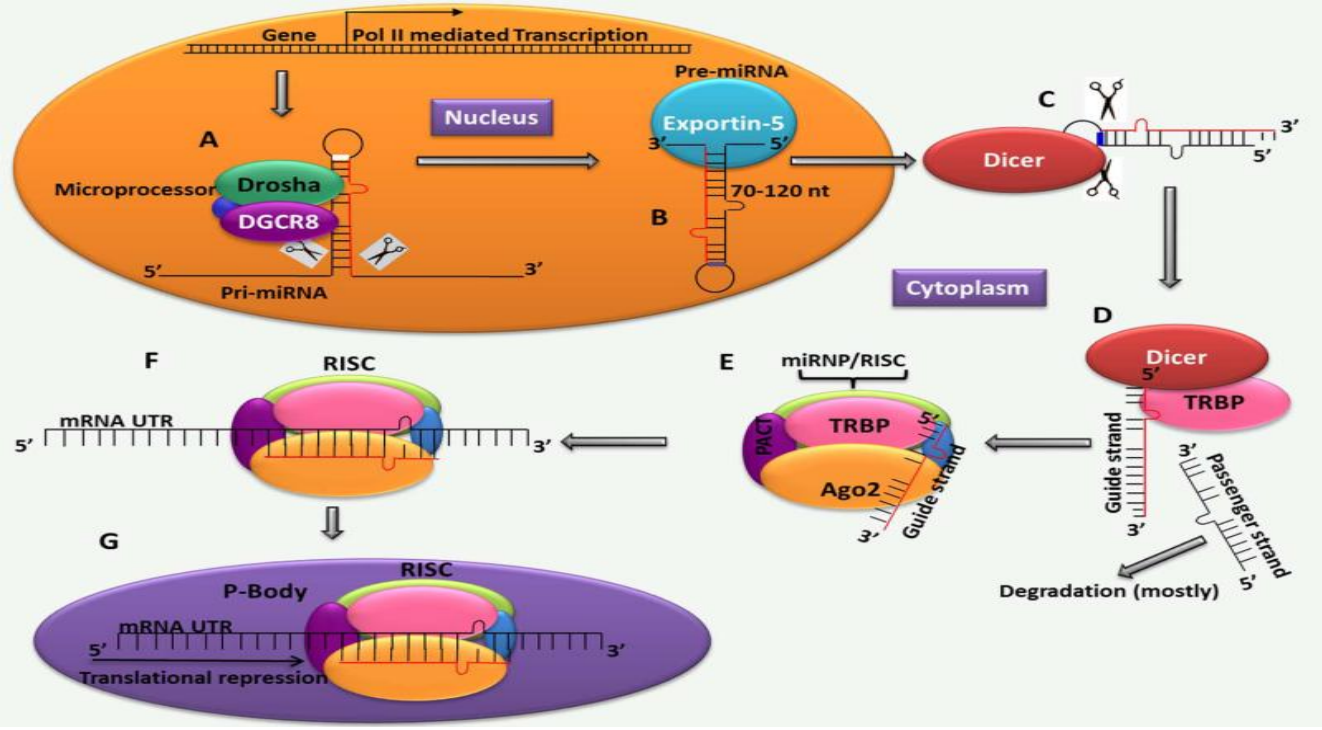

Figure 2.1: Canonical pathway of miRNA biogenesis (figure adapted and modified from (Yeung et al. 2005): **A)** miRNA gene in the nucleus is transcribed by RNA polymerase II or RNA polymerase III (in some cases) to form primary miRNAs of variable length with hairpin structures (pri-miRNAs). Processing of pri-miRNA is done by Microprocessor complex comprising of Drosha (classII RNaseIII enzyme) and DGCR8 (RNA binding protein) and the pri-miRNA is cleaved into 70-120 nucleotide long hairpin precursor form called premature (pre-miRNA) miRNA. **B)** these pre-miRNAs are trafficked outside the nucleus into the cytoplasm with the help of exportin-5. **C)** In the cytoplasm, DICER-I (RNAseIII endonuclease enzyme) cleaves the hairpin loop present in these pre-miRNAs and further processes these molecules into a mature 19-24bp long double stranded mature miRNAs with imperfect complementarity. **D and E)** the recruitment of Dicer-miRNA complex into the RNA-induced silencing complex (RISC) is facilitated by the RNA binding protein TRBP. RISC is a complex made up of protein kinase activator (PACT), Argonaute protein (Ago2) and other RNA binding proteins. **F)** with the association of mature miRNAs in to the microribonuclear protein complex (miRNP), the degradation of passenger strand takes place while the guide strand is directed to the 3'UTR of the target mRNA to cleave the target (in the event of perfect complementarity) or block the translation of the target mRNA (in case of partial complementarity between the target mRNA and the mature miRNA). **G)** Translational repression of mRNA targets is known to take place in the processing bodies (P –bodies) where the Ago2 protein along with the miRNA-mRNA complex is stored.

Argonaute (AGO) proteins to form a micro-ribonuclear protein complex (miRNP) also referred to as RNA-induced silencing complex (RISC) (**Figure 2.1E**) (Schwarz et al. 2003). It is still impossible to predict which strand of the miRNA duplex acts as a guide strand. However, many studies have suggested that the guide strand is mostly the strand with the most unstable 5' base pairing, while the strand with stable 5'end base pairing usually acts a passenger strand (the strand that is opposite to the guide strand) (Hibio et al. 2012). Passenger strand is also known as star miRNA (miR*). In most cases, they are degraded but sometimes, they can serve as functional miRNAs along with the guide strand by associating with AGO proteins (Czech et al. 2009; Okamura et al. 2009). Both the guide and passenger strands of the miRNA contains a seed region which

extends from 2nd nucleotide to the 8th nucleotide from the 5' end. This region is extremely important for the target recognition (Brennecke et al. 2005; Lewis et al. 2003). The complete sequence complementarity between the guide strand and the 3'UTR of the target mRNA directs the miRNP-RISC complex to the target site and promotes the Ago2 endonuclease-mediated cleavage of the target mRNA (**Figure 2.1F**). In most cases, translational repression of the target mRNA is due to a partial complementarity (if the complementarity exists only between the seed region of guide strand and 3' UTR of the target mRNA) between guide strand and the target. Multiple partial complementarity miRNA binding sites within the 3' UTR also leads to translational inhibition of the target mRNA (Bartel 2004; Yekta et al. 2004; Hutvágner & Zamore 2002). Apart from 3'UTR binding sites, miRNAs in some cases are also known to bind efficiently to the coding region or the 5' UTR sites in the target mRNA (Lytle et al. 2007; Tay et al. 2008). With this regard, it was also shown that mRNA cleavage can be triggered more effectively if miRNA finds its target sites on 3'UTR, whereas miRNA binding to the coding region can affectively block the translation (Hausser et al. 2013). Tay et al. 2008 showed that mouse genes responsible for regulating embryonic stem cell differentiation contained abundant miRNA binding sites in their protein coding region. mRNA targets bound to the miRNA along with Ago2 are localized to the processing bodies (GW/P-bodies) in the cytoplasm where they are either degraded or translationally repressed (**Figure 2.1G**) (Castilla-Llorente et al. 2012).

2.3 Regulatory functions of miRNA

Regulation of the target gene expression by the miRNA occurs mainly due to post-transcriptional repression of mRNA bound to the miRNA mediated by less efficient translational process or due to reduction in the mRNA levels mediated by RNA de-adenylation (Dalmay 2013; Wu et al. 2006; Eulalio et al. 2009; Eulalio et al. 2007; Giraldez et al. 2006). As mentioned earlier the extent of complementarity between the miRNA and the 3'UTR of the target mRNA is an important factor in determining the fate of the target where complete complementarity results in mRNA cleavage whereas, partial complementarity gives rise to translational repression (Hutvágner & Zamore 2002). Imperfect complementarity can also lead to accelerated degradation of the mRNA target triggered by the deadenylation of the mRNA (Wu et al. 2006). Two Mammalian miRNAs, *let-7* and miR-125b, with partial complementarity to 3'UTR of mammalian *lin-28* mRNA promoted the removal of poly(A) tail at the 3' end with the help of an exonuclease. This resulted in the rapid destabilization and accelerated decay of *lin-28* mRNA. However, the 5' cap was still retained (Wu et al. 2006). In the same study, it was also found that regulation of *lin-28* translation did not have any role to play in deadenylation rates and poly(A) tail was not really required for the repression of translation. Taken together, these examples demonstrate that the reduction in the protein output levels may not necessarily be due to the repression of the translational process but could also arise due to reduced mRNA levels caused by the destabilization or decay of mRNA targets (Guo et al. 2010). Canonical binding of miRNA (via seed region) to its target may not be

the only way by which miRNA functions. In some cases, miRNA may act as an RNA decoy for RNA-binding proteins that regulate mRNA translation (Eiring et al. 2010). For example, as shown in case of chronic myelogenous leukaemia (CML-BC), where miR-328 was observed to be significantly downregulated in myeloid blast cells. CML is a disease characterized by the enhanced activity of *BCR/ABL* oncoprotein, repressed translation of tumor suppressors and reduced granulocytic differentiation. Differentiation arrest in CML-BC is mediated by a poly(rC)-binding protein (*hnRNP E2*) which inhibits translation of *CEPBA* mRNA. It was shown that restoration of miR-328 expression levels resulted in interaction of miR-328 with *hnRNP-E2* in a non-seed sequence dependent manner thereby causing displacement of *hnRNP-E2* from *CEPBA* mRNA. This led to efficient translation of the released mRNA which further promoted the differentiation of myeloid progenitor cells. Alongside, a seed sequence dependent interaction of miR-328 with the 3'UTR of *PIM1* mRNA was observed. *PIM1* encodes for a survival factor. The resulting translational repression of *PIM1* mRNA led to decreased survival of leukemic blasts (Eiring et al. 2010). miRNAs in some rare occasions can perform dual functionality. i.e. they can activate or repress the translation of target mRNA. Under certain stressful conditions, cells growing in culture were shown to reversibly exit the cell cycle and become quiescent. Upon serum starvation, miR-369-3 bound to 3'UTR of *TNF-α* in a seed sequence-dependent manner and led to the translational upregulation of the target (Shobha Vasudevan, Yingchun Tong 2007). In this study, other well studied miRNAs including *let-7a* and synthetic miR*cxcr4* were used and miRNA target sites were placed in the 3'UTR of the reporter gene. Expected downregulation of the reporter gene was observed in the growing cells. However, the reporter was significantly upregulated in quiescent cells induced by serum starvation. In one of the studies, (Place et al. 2008) showed that miR-173 could induce the expression of the target genes, *E-Cadherin* and *CSDC2,* by directly targeting their promoters. But it is important to understand that miRNAs complementary to the promoter sequences of a particular gene may not always lead to transcriptional enhancement. This is because induction is dependent on intrinsic conditions such as DNA methylation of the targeted gene promoter (Li et al. 2006). miRNA mediated induction of the target genes also can be affected by distinct promoter environments across different cell types.

The exact mechanism by which miRNAs induce the expression of their target genes still remains unclear. Gene activation in such cases could be associated with chromatin changes upon miRNA binding (Li et al. 2006; Janowski et al. 2007). These authors discussed that miRNAs acted as transcription factors and these were complementary to certain motifs in the promoter region. It is well known that non-protein coding RNA expressed in the promoter region of a gene can repress its transcription (Petruk et al. 2006; Martens et al. 2004). Thus, miR-173 could have indirectly induced the expression of its target by directly targeting these motifs in the promoter region. From the above examples, It is clear that miRNAs also tune the molecular processes in a cell by regulating the expression of their target genes/mRNAs.

2.4 Viral miRNAs

During the course of evolution, several virus families have acquired small non-coding RNAs or miRNAs for pro-viral purposes (Grundhoff & Sullivan 2011). Viruses can use them to modulate theirs as well as host gene expression. Unlike the viral proteins, non-immunogenic miRNAs can target new transcripts by evolving rapidly. Viral miRNAs were first discovered in cells infected with Epstein-Barr virus (EBV). (Pfeffer et al. 2004). By now it is well known that miRNAs are encoded by a small number of RNA viruses and a large number of DNA viruses, particularly Herpesviruses (**Table 2**). Generally, the miRNAs belonging to the DNA viruses are validated, well an

Table 2: List of Viral mature miRNAs from miRBase 21 *http://www.mirbase.org*, (Kozomara & Griffiths-Jones 2014). The database contains 502 experimentally validated mature sequences of miRNAs encoded by 39 different viruses.

Viruses	Precursor miRNA	Mature miRNA
Bovine foamy virus	2	4
Bovine herpesvirus 1	10	12
Bovine herpesvirus 5	5	5
BK polyomavirus	1	2
Bovine leukaemia virus [K02120.1]	5	10
Bandicoot papillomatosis carcinomatosis virus type 2	1	1
Bandicoot papillomatosis carcinomatosis virus type 2	1	1
Duck enteritis virus	24	33
Epstein Barr virus [EMBL:AJ507799.2]	25	44
Herpes B virus [Refseq:NC_004812]	12	15
Human cytomegalovirus [EMBL:X17403.1]	15	26
Human herpesvirus 6B	4	8
Human immunodeficiency virus 1	3	4
Herpes Simplex Virus 1	18	27
Herpes Simplex Virus 2	18	24
Herpesvirus saimiri strain A11	3	6
Herpesvirus of turkeys	17	28
Infectious laryngotracheitis virus	7	10
JC polyomavirus	1	2
Kaposi sarcoma-associated herpesvirus [EMBL:U75698.1]	13	25
Mouse cytomegalovirus	18	29
Merkel cell polyomavirus	1	2
Mareks disease virus type 1 [EMBL:AF243438.1]	14	26
Mareks disease virus type 2	18	36
Mouse gammaherpesvirus 68 [EMBL:U97553.1]	15	28
Pseudorabies virus	13	13
Rhesus lymphocryptovirus	36	68
Rhesus monkey rhadinovirus [EMBL:AF210726.1]	7	11
Simian virus 40	1	2

-notated and widely accepted compared to the miRNAs from the RNA-viruses (Grundhoff & Sullivan 2011). It was believed that miRNAs could not be encoded by the genome of RNA viruses due to the fitness disadvantage imposed by the incurrence of cis cleavage of the viral genome or viral RNAs by miRNA processing machinery (Cullen 2010; Houzet & Jeang 2011). Nonetheless, studies have shown that several RNA viruses such influenza

and flavivirus can be genetically engineered to produce miRNA-like or active biological miRNA molecules (Rouha et al. 2010; Shapiro et al. 2010; Varble et al. 2010). In HIV, five virus encoded miRNAs were studied but were of low abundance to be widely accepted. They were poorly conserved across different strains and the results obtained from different labs were not consistent (Sun & Rossi 2011). Among several members of *retroviridae*, at least one of its members – bovine leukaemia virus (BLV) was reported to code for a miRNA sharing an identical seed sequence with the human miR-29a (Kincaid et al. 2012). Virus encoded miRNAs can be classified into two groups: 1) mimics of host miRNAs. 2) virus-encoded miRNAs.

2.4.1 Mimics of host miRNAs

A fraction of miRNAs encoded by viruses share their seed sequences with host miRNAs. It has been reported that three viruses namely Marek's disease virus 1 (MDV1), Kaposi's sarcoma associated herpes virus (KSHV) and BLV downregulate transcripts by docking at the same target sites as their counterpart miRNAs from the host (Grundhoff & Sullivan 2011; Kincaid et al. 2012). miR-K12-11 encoded by KSHV shares identical seed sequence with the human miR-155. Experimental studies have revealed that KSHV uses this orthologous miRNA to promote splenic B-cell expansion (Boss et al. 2011). Human miR-29 is also mimicked by the miRNAs encoded by EBV. Depending on the context and circumstance, miR-29 can function as a tumour repressor or as an oncogene and is strongly associated with tumorigenesis (Kincaid & Sullivan 2012). Virus-encoded miRNAs which mimic the host miRNA can potentially mediate the regulation of numerous transcripts containing the target sites which were generally targeted by the host miRNAs. Specific cellular functions can be affected by such regulation of regulatory networks. An example of such regulation is the inhibition of apoptosis in the infected cell. About 15% of viral miRNAs from viruses infecting humans share identical seed sequences with the host miRNAs and can mimic them (Kincaid & Sullivan 2012). Due to low abundance, unknown functionality and untested biogenesis, it is difficult to regard all annotated host and viral miRNA as bonafide miRNAs, just based on the seed matches between the viral and host miRNAs as they could come up by chance (Grundhoff & Sullivan 2011). This clearly suggests that only a small percentage of viral miRNA truly mimic the host miRNAs, but which ones this needs to be determined case by case.

2.4.2 Virus-encoded miRNAs

A miRNA encoded by Simian virus 40 (SV40) does not share any similarity with the human miRNAs. It has perfect complementarity to early viral transcripts and binds and targets them (reveiwed by Ghosh et al. 2009). This results in the disruption and reduced expression of early viral genes at later stages of lytic infection. Two non-coding RNAs (150-165nt) known as VA-RNAs (VA-RNA I and VA-RNA II) are transcribed in Adenoviruses. A gene encoding for VA-RNAI is present in all simian and human adenoviruses while only few viruses express a gene encoding for VA-RNAII. VA-RNAs are known to function as PKR (protein kinase R) inhibitor helping viruses

to overcome the shutdown of protein synthesis in host. PKR causes the phosphorylation of eIF2 (eukaryotic translation initiation factor) and thereby negatively regulates protein translation. With this phosphorylation, the affinity between eIF2α and eIF2B (guanine nucleotide exchange factor) is increased. This leads to the sequestration of eIF2B and the subsequent depletion of eIF2 bound to GTP to inhibit host mRNA expression. HAdV-C2 growth is significantly reduced upon VA-RNA I and II deletion. It has also been reported that highly abundant VA-RNAs bind to exportin-5. This inhibits the export of DICER mRNA and pre-miRNA to the cytoplasm by saturating export pathway dependent on exportin-5 to block the RNAi machinery (Lu & Cullen 2004; Bennasser et al. 2011). In the following section, some exemplary key functions performed by viral miRNAs will be discussed briefly.

2.5 Functions of viral miRNAs

Most functions associated to viral miRNAs can be divided into three main categories: 1) blocking apoptosis in the infected cells and prolonging cell longevity, 2) evading the host immune response, 3) limiting the viral lytic cycle by regulating viral or host genes. Detailed discussion of viral miRNA functions on each case is beyond the scope of this introduction, and is briefly summarized below with some examples.

2.5.1 Apoptotic inhibition of infected cells

Prolonging longevity of the infected cells by inhibiting cell death can be advantageous for viruses to establish persistent infections and to generate progeny particles. Apoptosis can be inhibited by the viral miRNAs targeting pro-apoptotic genes in the host cells infected by different viruses like EBV and KSHV (Kang et al. 2015). EBV is often associated with solid cell and B cell tumours. Infections with EBV can also give rise to mononucleosis, a hyperproliferative cellular disease (Kutok & Wang 2006). miR-BART5 encoded by EBV is known to target host pro-apoptotic gene PUMA (Choy et al. 2008). Another pro-apoptotic factor 'Bim' is also targeted by miRNAs belonging to EBV BART miRNA cluster (Marquitz et al. 2011). Additionally, miRNAs belonging to EBV BHRF1 cluster have been documented to play an important role in the inhibition of apoptosis of infected cultured B cells. KSHV encodes several miRNAs which can independently target host transcripts including apoptosis inducible receptor protein (TWEAKER) and block the initiation of cytokine signalling and caspase 3 activation all of which leads to the inhibition of apoptosis (Abend et al. 2010; Suffert et al. 2011). Pro-apoptotic factor BckAF1 is shown to be targeted by miRNAs encoded by three human herpesviruses (EBV, human cytomegalovirus [HCMV] and KSHV). These miRNAs dock at different target sites suggesting that BclAF1 is a major effector of herpesvirus life cycle and miRNAs binding to the target may not entirely depend on conserved target sequence (Ziegelbauer et al. 2009; Lee et al. 2012; Riley et al. 2012). Additionally, long 3' UTR of the abundantly expressed BclAF1 gene (more than 4kb) makes it a good target candidate to be regulated by miRNAs (reviewed in (Kincaid & Sullivan 2012).

2.5.2 Immune response evasion

Immune response evasion is important for viruses, in order to complete their infection cycle, and remain undetected by the host immune system (discussed in detail in part III of 1st chapter). miRNAs encoded by SV40 (miR-S1-5p and miR-S1-3p) have been well studied and provides a classic example where the virus use their miRNAs to evade the adaptive immune sensing in vivo. SV40 miRNAs targets early viral transcripts and promotes their cleavage thereby downregulating the expression of early viral genes during the latter stages of lytic infection (Sullivan et al. 2005). This notion is supported by observing greater cytotoxic T cell (CTL)-mediated lysis of the cells infected with SV40 miRNA mutant compared to the wild type SV40 infected cells. Several studies have shown that innate immune response mediated by the activation of natural killer cells (NK) *in-vitro* can be evaded by different human herpesvirus encoded miRNAs and star miRNA strand encoded by human JC polyomavirus (JCV) (Nachmani et al. 2009; Stern-Ginossar et al. 2009; Bauman et al. 2011). However, these findings require in vivo confirmation and validation to suggest that these viral miRNAs can successfully evade the NK activation of innate immune response. These findings suggest the possibility that viral miRNAs have a major role to play in immune response evasion.

2.5.3 Limiting the viral lytic cycle

Immune response in latently infected cells is mainly evaded by restricting the expression of viral proteins and thereby reducing viral antigenicity (Hilleman 2004). miRNAs encoded by different herpesviruses have been reported to play an important role in promoting the latent infection and regulating the transition between latent and lytic cycle (Murphy et al. 2008). The mechanism by which these miRNAs function in this regulation is reviewed in detail by (Grundhoff & Sullivan 2011). It is beyond the scope of this introduction and is briefly summarized below.

miR-K12-7-5p and miR-K-12-9-5p encoded by KSHV are known to target the transcripts belonging to lytic switch (RTA) protein (Bellare & Ganem 2009; Lin et al. 2011). Several other miRNAs encoded by KSHV promote enhanced latency by targeting some of the host transcripts (Lei et al. 2010; Liang et al. 2011; Lu et al. 2010). For example, host transcription factor (NFIB) was targeted by miR-K12-3-5p. NFIB is well known activator of RTA promoter (Lu et al. 2010). With this regard, enhanced lytic activity was observed in the experiments performed with a KSHV deletion mutant which reduced the maximum number of virus encoded miRNAs (Lei et al. 2010). As already discussed above, miRNAs encoded by other herpesviruses, although not as extensively studied as those of the KHSV, may play a major role in controlling the transition between lytic and latent infections. However, the biological relevance of the herpesvirus encoded miRNA targets was questioned in some studies as some of the transcripts inducing lytic replication of virus targeted by these miRNAs were undetectable in the RISC complex (Riley et al. 2012; Gottwein et al. 2011; Haecker et al. 2012). Still some quest

-ions remain for the viruses whose latent infection cycle is not well defined. In case of polyomaviruses the mechanisms behind the establishment of life long latent infections is not well defined. What is clearly known is, that miRNAs encoded by these viruses are known to regulate the expression of early genes during the latter stages of lytic infection (Sullivan et al. 2005; Seo et al. 2008; Seo et al. 2009; Chen et al. 2011; Cantalupo et al. 2005). Apart from this regulatory function, these miRNAs like the HzNV-1 and herpesviral miRNAs (as discussed above) could play a significant role in controlling the transition between the lytic and latent infection.

2.6 Host miRNAs and virus infection

There is evidence to suggest that miRNAs may be involved in inhibiting or boosting the infection and replication of some viruses like Hepatitis virus B and C (Jopling et al. 2005; Kitab et al. 2015). A majority of the miRNAs encoded by the host cells inhibits viral replication either by directly targeting the viral genome or indirectly by regulating a host factor associated with replication (Shrivastava et al. 2015). In this section, the well-studied role of host miRNAs in regulating infections mainly caused by HCV (hepatitis C virus) is summarized briefly.

Hepatitis C infection: Hepatitis C (HCV) virus are mainly responsible for acute and chronic infections in liver (Stauber 2000). miR-122 is highly expressed in liver cells and its interaction with HCV RNA positively regulates the viral replication in infected cells (Jopling et al. 2005; Randall et al. 2007). During this interaction two miR-122 copies bind to their respective target sites in the HCV genome at the 5'UTR (Li et al. 2014; Jopling et al. 2005). HCV translation is enhanced by the above-mentioned interaction which associates ribosomes to the HCV RNA at the time of translational initiation (Li et al. 2014). Additionally HCV RNA is protected from 5' exonuclease activity of Xrn1 (5' -> 3' exoribonuclease) by the miR-122-Argonaute protein complex which is bound to HCV genomic RNA at its 5'end (Shimakami et al. 2012; You Li et al. 2013). These observations clearly suggest that miR-122 plays a significant role in positive regulation of HCV replication (Masaki et al. 2015). Host miRNAs known to inhibit HCV RNA replication include let-7b, miR-196, miR-199a and miR-448 (Murakami et al. 2009; Cheng et al. 2012; Lagos-Quintana et al. 2002). miR-199a cancels out miR-122 effect by targeting the HCV genome at the 5'UTR just downstream to the miR-122 binding site and negatively regulates the HCV replication (Murakami et al. 2009). let-7b, expressed predominantly in spleen and liver tissues, targets HCV RNA by binding to different positions in the NS5B coding region and 5'UTR. As a result of this RNA genome of HCV is subjected to conformational changes and the replication of HCV is repressed (Cheng et al. 2012). miR-448 and miR-196 are also known to inhibit HCV replication by targeting the HCV RNA directly (Lagos-Quintana et al. 2002). In the recent study, miR-181c was found to negatively regulate viral replication by binding to the NS5A and E1 regions (Shrivastava et al. 2015) (Figure 2.5).

Other viral infections: In addition to HCV, role of host miRNAs in regulating other viral infections caused by hepatitis B (HBV), dengue virus (DENV) and human Immunodeficiency virus-1 (HIV-1) has been well studied. miR-199a-3p, miR-125a-5p and miR-210 negatively regulate the HBV surface antigen(HBsAg) expression and repress HBV replication (Xie et al. 2014; Zhang et al. 2010). miR-199a-3p in particular, binds the pre-S region in the HBV genome and the coding region of HBsAg. While miR125a-5p inhibit translation of the HBV genome by targeting HBsAg mRNA (Kitab et al. 2015; Zhang et al. 2010). miR122 which promotes HCV replication, shows opposite response to HBV infection. The activity of viral DNA polymerase is affected by miR-122 which negatively targets cyclin (G1). As a result the interaction between p53 and cyclin (G1) is interrupted which results in the positive regulation of HBV transcription inhibition mediated by p53 (Wang et al. 2012; Kitab et al. 2015). miRNAs expressed abundantly in resting CD4+T cells such as miR-382, miR-150, miR-28, miR-223 and miR-125b directly target the HIV-1 RNA at its 3'UTR (Huang et al. 2007). (Kakumani et al. 2013). miR-146a expression which was found to be upregulated upon DENV infection, was reported to enhance viral replication by inhibiting IFN-β secretion by targeting TRAF6 (TNF Receptor Associated factor 6)(S. Wu et al. 2013). miR-133a which is known to inhibit DENV replication by interfering with PTB (polypyrimidine tract binding protein) expression was found to be downregulated in Vero cells infected with four subtypes of DENV (DENV1-4) (Castillo et al. 2016). In one of studies, it was shown that 5' UTR consisting of stem loop promoter element (SLA) of the viral genome was targeted by miR-548g-3p thereby regulating DENV replication (Wen et al. 2015).

Part III – Innate immune sensing

Viruses and other pathogens can be sensed in the cytosol. This results in the induction of host innate immune response. Pathogen recognition receptors (PRRs) induce innate immune response in the host and are responsible for the cytosolic surveillance of pathogens and their clearance. Viral nucleic acids, carbohydrates and proteins with conserved features and non-resident RNA molecules represent PAMPs (pathogen associated molecular patterns). PRRs recognizing PAMPs can be classified into two subgroups: i) Extracellular PRRs, ii) Intracellular PRRs, based on their location in the cell (Girardin et al. 2002). Extracellular PRRs are predominantly expressed on the endosomal or plasma membranes of the immune cells like dendritic cells and macrophages and include CLRs (C-type lectin receptors) and TLRs (Toll-like receptors) (Takeuchi & Akira 2010; Dambuza & Brown 2015; Girardin et al. 2002; Opitz et al. 2007; Kai Chan & Gack 2016) (**Figure 3.1**). Extracellular PAMPs are typically sensed by these receptors (Thompson et al. 2011; Opitz et al. 2007; Girardin et al. 2002). Intracellular PRRs, on the other hand are found in the nucleus or cytoplasm of the mammalian cells. The members of intracellular PRRs include RLRs (RIG-I like receptors), NLRs (NOD like receptors) also referred as leucine rich nucleotide binding domain, cGAS (DNA sensor marked by the presence of cyclic GMP-AMP synthase) and IFI16 (IFNγ inducible protein 16 (Goubau et al. 2013; Meylan et al. 2006; Sparrer & Gack 2015) **(Figure 3.1**). Ubiquitous expression of these intracellular PRRs ensures efficient recognition of viral pathogens in the nucleus or the cytoplasm of the mammalian host cell. PRRs activate specific adaptor proteins such as MYD88 (Burns et al. 1998; Wesche et al. 1997) or STING (IFN stimulator genes) (Ishikawa & Barber 2008; Zhong et al. 2008) and MAVS (mitochondrial antiviral signaling protein) (Seth et al. 2005) following the PAMPs recognition to induce innate immune signalling (Kato et al. 2005; Chow et al. 2015) (**Figure 3.1**).

Several host defence molecules such as pro-inflammatory chemokines and cytokines, type I IFN and type III IFNs are produced following the signal transduction process initiated by PRR activation (Kagan & Barton 2015; Mogensen 2009; Brubaker et al. 2015). IFNs released by the host cells bind to the IFN receptors (IFNAR1/IFNAR2) in a paracrine or autocrine manner and stimulate the expression of ISGs (IFN-stimulated genes) (Schneider et al. 2014). ISG encoded proteins confer antiviral state by carrying out crucial functions such as cytokine production, targeting or blocking important steps in virus life cycle and regulating innate sensing. Chemokines and cytokines produced as a result of PRR activation also play a crucial role in tuning adaptive immune response (Schoggins & Rice 2011; Wong & Chen 2016). However, Host innate immune responses described above potently inhibit virus propagation and viruses time try to evade these responses by shielding their nucleic acid structures through structural modifications and by avoiding exposure of the viral genomes in the cytoplasm (Fields et al. 2007; Witteveldt et al. 2014). Additionally, viruses can facilitate the cleavage or degradation of the adaptor protein or PRRs or induce the relocalization or seclusion of PRRs (X.-D. Li et al. 2005; K. Li et al. 2005; Huang et al. 2014; Kai Chan & Gack 2016). In this part, I would like to review the

role of cytosolic DNA and RNA sensors in the host innate immunity. Additionally, I would also like to describe the steps taken by viruses (with examples) to block the sensor dependent innate immune activation.

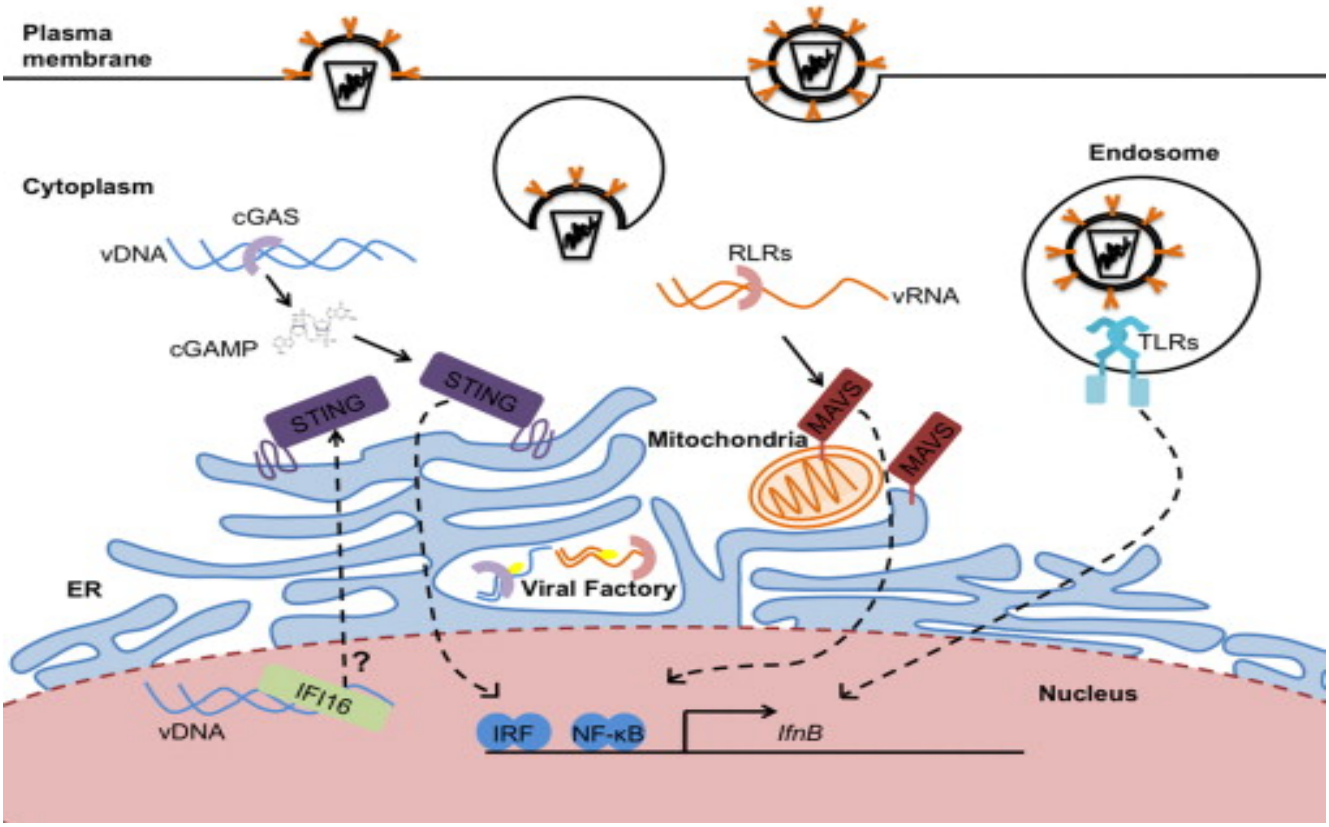

Figure 3.1: Induction of Innate immune signaling by PRRs initiating the signal transduction process and type I IFN production (Chow et al. 2015). Viruses enter the host cell by endocytosis or by direct passing through the plasma membrane barrier. Viral genomes exposed in the endosome are recognized by TLRs. RLRs can recognize the viral RNA in the cytoplasm and cause the induction of Type I IFN through the activation of MAVS expressed on ER membranes, mitochondria and peroxisomes. Viral DNA can be sensed by nuclear IFI16 or cytosolic cGAS. STING activated by both these sensors initiates signalling cascade that causes the secretion of type I IFNs. Replication intermediates of viruses present in viral factories (intracellular inclusion compartments) can be recognized by RLRs and cGAS. All the above-mentioned pathways can lead to enhanced transcription of IFNβ facilitated by NF-kB and IRF3 activation. TLRs, toll-like receptors, RLRs, RIG-I like receptors; MAVS, mitochondrial antiviral signaling protein; ER, endoplasmic reticulum; *IFI16, IFNγ inducible protein 16; cGAS, cyclic GMP-AMP synthase;* STING *stimulator of IFN genes; IFN*β, interferon β, *NF-KB, nuclear factor kappa-light-chain-enhancer of activated B cells; IRF, Interferon regulatory factor*

3.1 Role of RNA sensors

Genomic RNA of the viruses infecting the host can be detected by cytosolic RNA sensors (Goubau et al. 2013; Jensen & Thomsen 2012; Gantier & Williams 2011; Wilkins & Gale 2010). A large number of these sensors have been identified in the recent past (discussed in the section below). Additionally, many studies have also reported the activation of innate antiviral immune response by non-self dsRNA molecules

(Wang et al. 2016; Gantier & Williams 2007; Creagh & O'Neill 2006; Marques et al. 2006). Apart from Genomic RNA, transcription products or replication intermediates can also be sensed and thereby trigger type 1 IFN response (Iwasaki 2012; Lande & Gilliet 2010; Pichlmair et al. 2006; Hornung et al. 2006; Triantafilou et al. 2012). Inflammasome activation and IFN induction can also be triggered by the sensing of ssRNA (single stranded RNA) by some members of the NLR (NOD-like receptors) family (Iwasaki & Medzhitov 2010; Poeck et al. 2010; Wen et al. 2013; Kanneganti 2010). Viral genomic dsRNAs or non-self dsRNAs (dsRNAs not found in the host) are typically sensed by LGP2 (DHX58), MDA5 (IFIH1 also known as melanoma differentiation associated protein 5) and RIG-I (retinoic acid-inducible gene-I protein) which constitute the three key members of RLRs. Most cell types express these sensors. These proteins contain carboxy-terminal domain and helicase domain at the centre and these domains are implicated in the binding of dsRNA (Yoneyama et al. 2004; Andrejeva et al. 2004) (**Figure 3.2**). Both **MDA5** and **RIG-I** make use of CARD signaling domain (caspase activation and recruitment domain) to induce antiviral immune signaling upon sensing of viral RNA. RIG-I signaling is negatively regulated by LGP2 which does not contain a CARD domain. However, LGP2 initiates RNA binding to MDA5 and thus LGP2 usually has a positive effect on MDA5-mediated dsRNA sensing (Bruns & Horvath 2015). Unique features of viral RNA (usually not found in mature RNA molecules of the host) are recognized by MDA5 and RIG-I (discussed in detail in the section below). Functional importance of these sensors in viral RNA sensing has been demonstrated in several studies studying the effect of virus infection in mouse and human cells lacking functional MDA5 and/or RIG-I (Errett et al. 2013; Gitlin et al. 2006; Kato et al. 2006; Kato et al. 2005). Several minus strand RNA viruses (arenaviruses, influenza viruses and rhabdoviruses) and plus strand RNA viruses (HCV and JEV) can be sensed by RIG-I (Goubau et al. 2013; Kato et al. 2006; Loo et al. 2008). On the contrary, picornaviral RNA is sensed by MDA5 (Barral et al. 2009; Gitlin et al. 2006; Deddouche et al. 2014). Additionally MDA5 and RIG-I have been reported to recognize a large number of other viruses, including west Nile virus, dengue virus and paramyxoviruses (Loo et al. 2008). Diverse RNA species generated upon infection by the above mentioned viruses were also reported to be sensed by these sensors in a temporal dependent manner (Goubau et al. 2013; Schlee 2013). Additionally, significant number of DNA viruses (Adenoviruses, KSHV, HSV-1 and EBV) are detected by RIG-I (Goubau et al. 2013; Rasmussen et al. 2009; West et al. 2014). RNA species produced by RNA polymerase III are sometimes also sensed by RIG-I.

Host enzymes and post translational modifications (PTMs) regulate the signal transduction process initiated by MDA5 and RIG-I (Chiang et al. 2014). C-terminal domain and CARDs are constitutively phosphorylated in the non-infected cells at specific threonine or serine residues. This allows the proteins to be kept in the repressed or inactive signaling state (Gack et al. 2010; Maharaj et al. 2012; Wies et al. 2013; Sun et al. 2011; Takashima et al. 2015) (**Figure 3.2**). Additionally, auto-repressed conformation of RIG-I is maintained by intramolecular interaction between CARDs and the helicase domains (Kolakofsky et al. 2012; Saito et al. 2007). However, MDA5 in the non-infected state is kept in a open conformational structure (Berke & Modis 2012). RNA binding

to these sensors promotes the binding of PACT (protein kinase R activator) (Kok et al. 2011) and stimulates its ATPase activity causing conformational changes in MDA5 and RIG-I (Kowalinski et al. 2011). CARDs become free to interact with other regulatory molecules as a result of this conformation change. Phosphatase PP1: PP1γ or PP1α isoform which causes the dephosphrylation of CARDs is recruited by MDA5 and RIG-I (Wies et al. 2013). E3 ubiquitin ligases RNF135 (also known as Riplet) and TRIM25 (tripartite motif protein) are recruited by RIG-I to facilitate the attachment of Lys63-linked ubiquitin polymers to C-terminal domain and CARDs (Gack et al. 2007; Oshiumi et al. 2013). RIG-I undergoes tetramerization and interacts with MAVS expressed on mitochondrial outer membrane and other membranes associated to mitochondria (MAMs) as a result of this Lys63-dependent ubiquitylation process (Gack et al. 2007; Horner et al. 2011; Zeng et al. 2010). It has been shown that RIG-I signaling can be repressed by DUBs (deubiquitylating enzymes), CYLD, USP3 and USP21 which facilitates the removal of Lys63-linked ubiquitin polymers (Chiang et al. 2014). Mitochondrial targeting chaperone protein involved in trafficking, 14-3-3e was recently shown to induce the translocation of RIG-I upon activation to the mitochondria for it to interact with MAVS (Liu et al. 2012). This translocation was mediated by the interaction of 14-3-3e with RNA in complex with TRIM25 and RIG-I (Liu et al. 2012; Gack et al. 2007). The requirement of Lys63-ubiquitination for MDA5 signaling and multimerization is still a matter of debate, as It has been shown that MDA5 filaments, which bind to MAVS and represent the activated form of MDA5, can be formed just upon binding of MDA5 to long dsRNA (B. Wu et al. 2013). Apart from MAMs and mitochondria, MAVS are also expressed in peroxisomes and here it triggers downstream signaling which results in the induction of type III IFN genes (Dixit et al. 2010). Upon activation, MAVS aggregates to form filamentous structures which looks like prions (Hou et al. 2011). This causes the assembly of large multiprotein 'signalosome' complex (Pomerantz & Baltimore 1999; Jacobs & Coyne 2013) consisting of IKKe (IkB kinase-e) or TBK1, ternary complex of IKKα-IKKγ-IKKβ, and TRAF (TNF receptor associated factor) proteins. This signalosome complex promotes IRF3 (Interferon regulatory factor -3) or IRF7 and NF-kB activation (Oganesyan et al. 2006; Fitzgerald et al. 2003; Michallet et al. 2008; Guo & Cheng 2007). AP1 (activator protein 1) together with NF-KB, IRF3 and IRF7 induces the enhanced expression of IFN genes, ISGs and cytokines (IL-6, TNF and IL-8) which confers antiviral state in the non-infected host cells neighbouring the infected cell (Panne et al. 2007; Wertz & Dixit 2010). All of the above-mentioned processes are summarized in **Figure 3.2**. Interestingly, MDA5 and RIG-I were reported to inhibit the replication of HBV (Hepatitis B virus) and Influenza virus without inducing the expression of cytokine genes but by blocking the interaction of viral RNA with viral proteins (Sato et al. 2015; Weber et al. 2015; Yao et al. 2015).

3.1.1 Other RNA sensors

OAS (oligoadenylate synthase): 2'-5' oligoadenylate produced by OAS leads to the activation of endoribonuclease RNaseL. This leads to the dimerization of RNaseL. Both, viral and cellular RNAs are degraded

into smaller fragments by the activated RNaseL which causes the repression of virus propagation (reviewed in (Hornung et al. 2014). These fragments can also serve as PAMPs to be sensed by RIG-I and induce the production of IFNs.

PKR (Protein kinase R): viral dsRNA sensed by PKR blocks cap-dependent translation mediated by the phosphorylation of eIF2α (eukaryotic initiation factor 2α) (Garcia et al. 2007) and induces the activation of autophagy and inflammasomes (reviewed by (Dalet et al. 2015; Lu et al. 2012; Siddiqui & Malathi 2012). In a recent study, stress granules were reported to be formed as a result of PKR induction. Enhanced interaction between viral RNA and RLRs can be facilitated by these stress granules (Lloyd 2013).

DExD/H helicases (non RLRs): Besides RLRs, large number of DExD/H helicases have been shown to work as RNA sensors (Fullam & Schröder 2013). Additionally, these proteins were shown to have immunoregulatory functions and be involved in RNA metabolism in host cells (Schröder 2011; Fuller-Pace 2006). DDx3 for example, performed the role of a viral RNA sensor and caused the induction of IFNβ through the activation of MAVS. additionally, translation of PACT mRNA was promoted by the regulation of IKKe and TBK1 activity mediated by DDX3 (Goubau et al. 2013). Viral RNA was shown to be degraded by DDX60 which served as a cofactor in RIG-I activation (Oshiumi et al. 2015).

3.2 Role of DNA sensors

Innate immune response induced upon cytosolic recognition of foreign DNA in mammalian cells has been well studied over a past few years. A large number of DNA sensors and adaptor proteins have been identified in the recent past (Dempsey & Bowie 2015) (discussed in the section below). STING is a key adaptor protein employed by most of these sensors (Zhong et al. 2008; Ishikawa et al. 2009; Sun et al. 2009). Functional relevance of other DNA sensors still needs further validations, but good evidence exists that IFI16 and cGAS serves as a bonafide receptor for sensing viral DNA. cGAS recognizes the dsDNA from certain viruses by binding to it in the cytoplasm. This causes the activation of cGAS (Sun et al. 2013; J. Wu et al. 2013). The viruses sensed by cGAS include retroviruses whose DNA is generated from their RNA genomes via reverse transcription process and other DNA viruses (**Figure 3.3**) (Sun et al. 2013; D. Gao et al. 2013; X.-D. Li et al. 2013; Schoggins et al. 2014). cGAMP (cyclic GMP-AMP) which is cyclic dinucleotide is produced by cGAS upon its activation (J. Wu et al. 2013; P. Gao et al. 2013; Ablasser, Goldeck, et al. 2013; Diner et al. 2013; Zhang et al. 2013). cGAMP has a characteristic 2'-5' phosphodiester bond which resembles 2'-5' oligoadenylate synthesized by OAS (oligoadenylate synthase) (Ablasser, Goldeck, et al. 2013). cGAMP then binds to STING and causes its activation. At the same time, cGAMP can translocate via gap junctions to the neighbouring non-infected cells and thereby activate STING in those cells thus alerting them of virus infection (Ablasser, Schmid-Burgk, et al. 2013). Interestingly, several studies have shown that IFI16 was localized in the nucleus of many cell types (Kerur et al. 2011; Veeranki & Choubey 2012; Unterholzner et al. 2010). Here, it was able to different

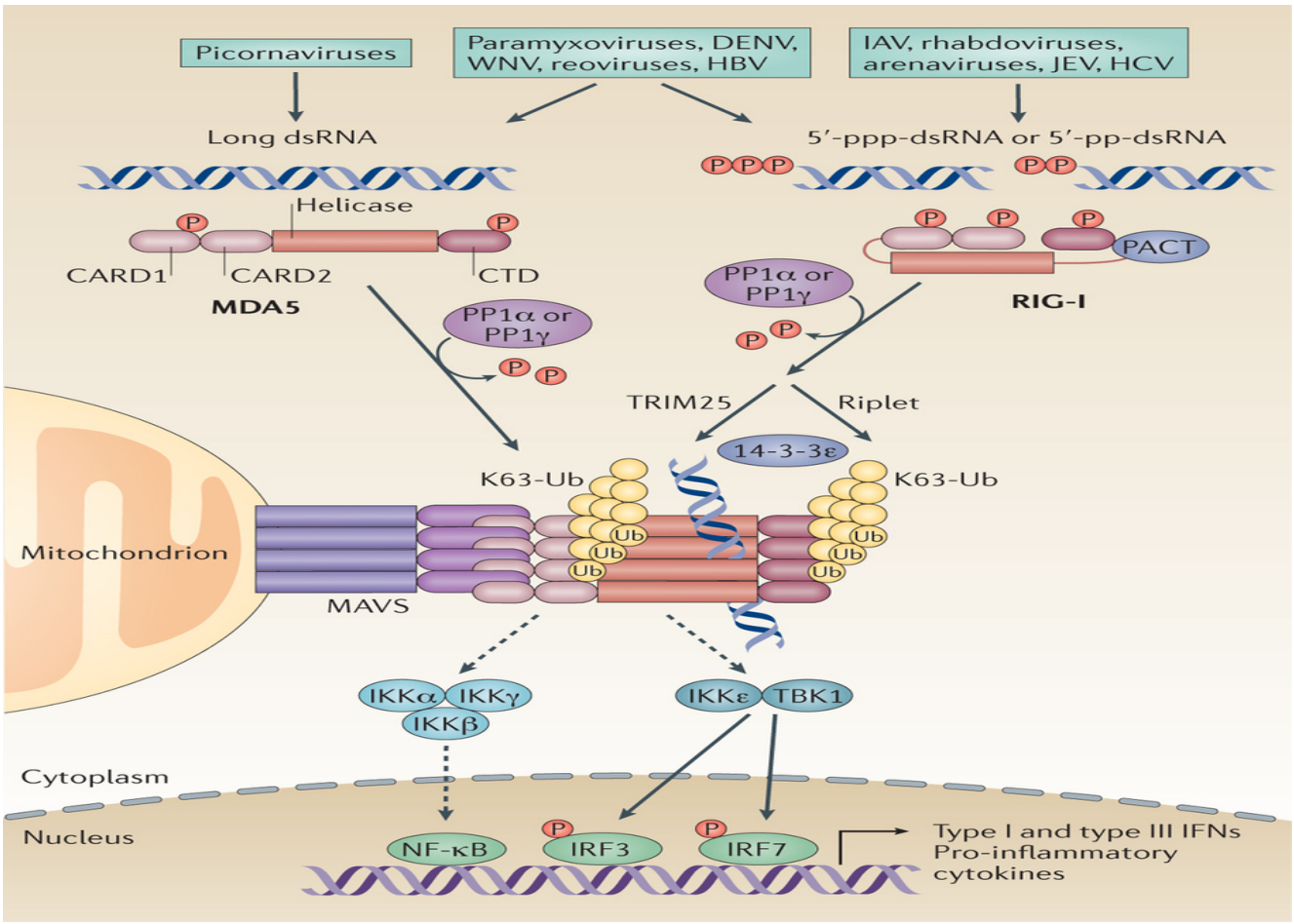

Figure 3.2: Role of RLRs (RIG-I like receptors) as cytosolic RNA sensors (Kai Chan & Gack 2016). RLRs are RNA sensors which can detect foreign RNA or viral RNA and their replication intermediates. MDA5 and RIG-I represent the two important members of RLRs. CTDs (carboxy terminal domains) and CARDs (caspase activation and recruitment domains) of RIG-I and MDA5 are phosphorylated in the non-infected cell which makes them inactive. Additionally, closed conformational state of RIG-I also maintains the protein in a repressed state. Upon viral infection, long dsRNA is sensed by MDA5 while short dsRNA molecules containing 5' diphosphate or 5' triphosphate groups are recognized by RIG-I. PP1 proteins (PP1γ or PP1α isoform) dephosphorylates MDA5 and RIG-I and activates the CARD domains following the RNA binding. With the help of E3 ubiquitin ligases (RNF135 and TRIM25), CARDs and CTD domains both undergo Lys63-linked ubiquitylation to further activate RIG-I leading to the formation of RIG-I tetramers. This represents active signaling form of RIG-I which interacts with MAVS adaptor (mitochondrial antiviral signaling) protein expressed abundantly on membranes associated to mitochondria (MAMs), mitochondria or peroxisomes (not displayed). Mitochondrial protein 14-3-3e functions as chaperone to transport RIG-I towards MAVs. For MDA5, formation of MDA5 filaments takes place upon long dsRNA binding which initiates the binding of MDA5 and MAVS. MAVS activation also results in the activation of IKKα-IKKγ-IKKβ complex, IKKe and TBK1 which in turn phosphorylates NF-kB, IRF3 and IRF7. AP1 (activator protein 1) (not shown here) together with NF-KB, IRF3 and IRF7 induces the enhanced expression of type 1 and type III IFNs. Several proinflammatory chemokines and cytokines are induced by IFN to confer antiviral state. Direct signaling events are indicated by solid arrows while indirect signaling events are illustrated by dashed arrows. *MDA5, melanoma differentiation associated protein 5; and RIG-I, retinoic acid-inducible gene-I protein; PP1, protein phosphatase 1; TRIM25, tripartite motif protein; RNF135, ring finger protein 135; Ub, ubiquitin; P, phosphate; JEV, Japanese encephalitis virus; HCV, hepatitis C virus; DENV, dengue virus; WNV, West Nile virus; PACT, protein kinase R activator; K63-Ub, Lys63-linked ubiquitylation; IAV, influenza A virus; HBV, hepatitis B virus; NF-KB, nuclear factor kappa-light-chain-enhancer of activated B cells; IRF, Interferon regulatory factor; IKK, inhibitor of nuclear factor kappa-B kinase; TBK1, tank binding kinase 1.*

-iate between resident and foreign DNA. Since many DNA viruses (polyomaviruses, papillomaviruses and herpesviruses, adenoviruses) replicate in the nucleus, these viruses can potentially be sensed by nuclear IFI16 (Conrady et al. 2012; Costa et al. 2011; Lo Cigno et al. 2015) . However, the exact mechanism of viral DNA recognition by IFI16 in the nucleus and the process which causes the activation of TBK1-STING-IRF3 axis in the cytoplasm need further investigations and validations.

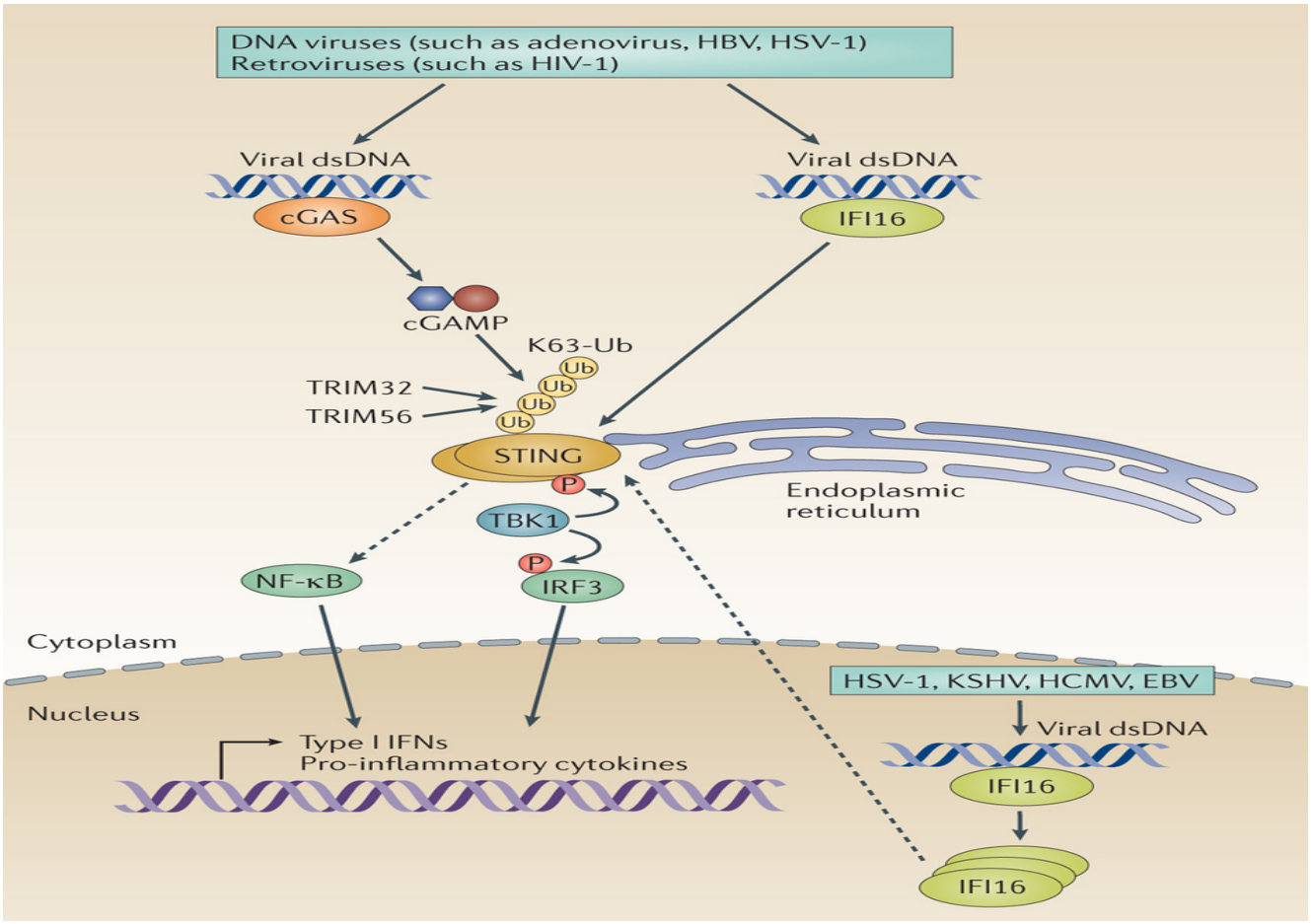

Figure 3.3: DNA sensors, IFI16 and cGAS recognize viral DNA and induce antiviral signaling (Kai Chan & Gack 2016). dsDNA generated from RNA genomes of retroviruses upon reverse transcription or dsDNA of DNA viruses are recognized by cytosolic cGAS in the infected cells. cGAS binds the viral DNA and becomes activated to produce second messenger cGAMP. cGAMP then activates STING by binding to it in the endoplasmic reticulum and this initiates signalling pathway that induces the expression of IFN genes. The activation of STING is further enhanced by its dimerization. TRIM56 and TRIM32 links Lys63-linked polyubiquitin chains to the STING dimers. Additionally, TBK1 phosphorylates STING. IFI16 (IFNγ inducible protein) detects viral dsDNA in the nucleus as well as in cytoplasm. IFI16 multimerizes to form filaments after binding to the viral DNA and signals to STING localized in the cytoplasm. Activated STING then induces the activation of IRF3-TBK1 axis and NF-kB which leads to the enhanced expression of pro-inflammatory cytokines and type 1 IFN genes. Established and validated signaling events are indicated by solid arrows while indirect signaling events which requires further validations are illustrated by dashed arrows. *cGAS, cyclic GMP-AMP synthase; cGAMP, cyclic guanosine monophosphate-adenosine monophosphate; STING, stimulator of interferon genes; TRIM, tripartite motif protein; TBK1, tank binding kinase 1; IFI16, IFNγ inducible protein 16; IRF, Interferon regulatory factor; NF-KB, nuclear factor kappa-light-chain-enhancer of activated B cells; Ub, ubiquitin; KSHV, Kaposi sarcoma-associated herpesvirus; HSV-1, herpes simplex virus 1; HBV, hepatitis B virus; P, phosphate; K63-Ub, Lys63-linked ubiquitylation; HCMV, human cytomegalovirus; EBV, Epstein–Barr virus.*

3.2.1 Other DNA sensors

AIM2 (Absent in melanoma 2): AIM2 belongs to PYHIN (pyrin and HIN200domain containing protein) family of proteins (Burckstummer et al. 2009; Fernandes-Alnemri et al. 2009; Hornung et al. 2009) and has been shown to function as viral DNA sensor (Rathinam & Fitzgerald 2010; Muruve et al. 2008). AIM2 induces caspase 1 dependent Inflammasome activation thereby giving rise to interleukin (IL-18 and IL-1β) production (Hornung et al. 2009). Some DNA viruses including mouse cytomegalovirus and vaccinia virus cannot be sensed in AIM2 deficient host cells (Rathinam et al. 2010).

RNA polymerase III: Apart from its main role in small RNA synthesis, RNA polymerase III localized in the cytoplasm was reported to convert viral dsDNA with AT-rich sequences into 5'-triphosphate group containing small RNAs which then activate RIG-I (Dempsey & Bowie 2015).

DAI (DNA-dependent activator of IFN-regulatory factors): DAI is considered to be the first viral DNA sensor discovered in the cytoplasm (Wang et al. 2008; Takaoka et al. 2007). It recognizes AT-rich DNA (poly dA:dT) (Ablasser et al. 2009). Type 1 IFNs are produced as a results IRF3 and NF-kB activation by DAI. However, some of DAI deficient human and mouse cells still showed active immune response upon exposure to viral DNA suggesting a cell specific or redundant role of DAI (Dempsey & Bowie 2015).

Others: other DNA sensors or sensor like molecules include DNA-PK (DNA-dependent protein kinase), DDX41, DHX36, MRE11 and DHX9 (reviewed by (Dempsey & Bowie 2015).

NLRs (NOD-like receptors): PAMPs or danger signals that are produced upon microbial infection are recognized by NLRs (reviewed by T. D. Kanneganti, Lamkanfi, & Núñez, 2007). The human genome encodes 22 NLRs (Zhong et al. 2013). Out of those, NOD2 (nucleotide-binding oligomerization domain-containing protein 2) and NLRP3 (pyrin domain–containing 3) are well characterized with respect to sensing viral nucleic acids and viral infection (Wen et al. 2013; Kanneganti 2010). Following infection with IAV and RSV (respiratory syncytial virus) or ssRNA transfections, activated NOD2 initiates type 1 IFN production (Sabbah et al. 2009; Pandey et al. 2009). MCV and IAV infections triggered NLRP3 inflammasome activation (Allen et al. 2009; Ichinohe et al. 2009; Poeck et al. 2010).

3.3 Viral PAMPs

Ligands sensed by RLRs: Poly(I:C) (polyinosinic-polycytidic acid), an analogue of synthetic dsRNA can be sensed by RIG-I and MDA5. However, RIG-I specifically recognizes short dsRNA while MDA5 senses high molecular weight poly(I:C) (Goubau et al. 2013; Kato et al. 2006; Schlee 2013). RIG-I specifically recognizes 5'-diphosphate or 5'-triphosphate groups which is generally absent in mature cellular RNAs (Hornung et al. 2006; Pichlmair et al. 2006; Goubau et al. 2014). Additionally, RIG-I can recognize particular structures like panhandle

or hairpin conformations which exists in several viruses with RNA genomes (for example Influenza A virus). Specific sequence signatures like poly-UC or poly-U motifs (generally observed in HCV RNA) further enhance RIG-I recognition (Saito et al. 2008; Uzri & Gehrke 2009). 5'-e-region found in pre-genomic RNA of HBV (hepatitis B virus) was shown to be sensed by RIG-I (Sato et al. 2015). It was also reported that RIG-I was able to detect genomic RNAs and RNA arising from defective IAV (influenza A virus) and Sendai virus particles (Baum et al. 2010; Rehwinkel et al. 2010). In addition to high molecular weight-poly(I:C), large RNA aggregates which have a web like appearance generated upon EMCV (encephalomyocarditis virus) infection stimulate the activity of MAD5 (Pichlmair et al. 2009). dsRNA and ssRNA regions are contained within these aggregates. Apart from this, the presence of ribose2'-O-methylation at the 5'cap of RNA fail to activate MDA5 (Zust et al. 2011). Such structures are prevalent in mRNAs of higher eukaryotes and they help to differentiate between the self and foreign RNA from MDA5 recognition.

Ligands sensed by cGAS and IFI16: dsDNA genome of many DNA viruses are recognized by cGAS in a sequence independent manner (Civril et al. 2013; Wu & Chen 2014; Cai et al. 2014; Xiao & Fitzgerald 2013). cGAS is activated by binding directly to DNA by forming hydrogen and electrostatic bond interaction with sugar phosphate backbone (Hornung et al. 2014; Cai et al. 2014). dsDNA generated from RNA genomes of HIV-1 and other retroviruses upon reverse transcription are recognized by cGAS (D. Gao et al., 2013; Lahaye et al., 2013; Rasaiya-ah et al., 2013). Interestingly, sequence dependent activation of cGAS by HIV-1 sstDNA (strong-stop DNA) was reported (Herzner et al. 2015). On the contrary, IFI16 most often localized to the nucleus, recognizes the DNA from KSHV (Kaposi sarcoma-associated herpesvirus) and HSV-1 (herpes simplex virus 1) in the nucleus (Orzalli et al. 2012). Additionally, IFI16 has been reported to bind to the secondary structures present in HIV-1 ssDNA (Unterholzner et al. 2010; Orzalli & Knipe 2014). Viral DNA may be sensed cooperatively by IFI16 and cGAS and induce innate immune signaling (Orzalli et al. 2015).

PAMPs sensed by NLRs: viral ssRNA is recognized by NOD2, while other PAMPs like viral DNA or RNA in complex with ATP and ROS (reactive oxygen species) are mainly recognized by NLRP3 (Sabbah et al. 2009; Allen et al. 2009; Ichinohe et al. 2009; Muruve et al. 2008; Kanneganti et al. 2006)**.** The association between NOD2 and MAVS induces type 1 IFN production (Sabbah et al. 2009). NLRP3 inflammasomes on the other hand, associates with ASC (apoptosis-associated speck-like protein) protein to produce active caspase 1. Active caspase 1 cleaves IL-1β and IL-18 cleaves IL-18 and IL-1β to their mature form (Silva et al. 2013; Lamkanfi et al. 2007; Martinon et al. 2002; Dowds et al. 2003; Franchi et al. 2009).

3.4 Evasion of innate immune sensing by viruses

Viral pathogens have developed strategies to evade or suppress intracellular PRRs activation, in order to complete their infection cycle in the host. Here, we briefly summarize molecular strategies employed by the viruses (with some examples) to avoid activation of MAVS and RLRs (mostly by RNA viruses), cGAS, IFI16 and

STING (mostly by DNA viruses and some RNA viruses). Downstream molecules, such as NF-kB, IRF3, TBK1 and IRF7 shared between PRRs and RLRs which helps trigger the innate immune response are inhibited by many viruses. Additionally, some viruses are also known to suppress the activity of certain antiviral effector proteins or block type 1 IFN receptor signaling. The detailed review of such strategies used by viruses is beyond the scope of this introduction. Details are explained in detail in the review article by (Bowie & Unterholzner 2008; Taylor & Mossman 2013).

Viral genome sequestration: Several viruses can escape from being sensed by RLRs by sequestering their genomes (**Figure 3.4**). Viruses can trigger the assembly of specific replication compartments inside the cellular membranes or could replicate on subcellular organelles like mitochondria, Golgi apparatus or endoplasmic reticulum (ER) (Ravindran et al. 2016; Schmid et al. 2014) to ensure that viral RNA is prevented from being accessed by RLRs. For instance, dsRNA of DENV (Dengue virus, a member of flaviviridae) was efficiently concealed from cytosolic PRRs by replicating in the ER convoluted membranes (Uchida et al. 2014). IAV, another RNA virus is prevented from being sensed by cytosolic RLRs by replicating in nucleus (Killip et al. 2015). Although IAV travels for a short time through the cytoplasm, it has developed strategies to escape from being recognized. For example, NPs (nucleoproteins) of some of the IAV strains encapsidate their RNA genome and with the help of PB2 subunit of viral polymerase. This way virus avoids being recognized by RIG-I (Weber et al. 2015). Strong affinity between NP and Lys-627 residue of PB2 allows the viral genome to be tightly packed and thereby blocks the RIG-I binding to the genome.

Likewise, other viruses also make use of host or virus encoded proteins to shield their genome from being recognized by RLRs. For instance, vaccinia virus-E3 protein, Marburg virus and EBOV (Ebola virus)- VP35 (viral protein 35), and IAV-NS1 (non-structural protein-1) are known to bind viral dsRNA genome to inhibit RIG-I recognition (Cárdenas et al. 2006; Ramanan et al. 2012; Hatada & Fukuda 1992; Qian et al. 1995; Donelan et al. 2003; Valentine & Smith 2010). On the contrary, host cell encoded, RNA binding protein-La is used by RSV to avoid RIG-I binding to viral RNA (Bitko et al. 2008). Finally, HPIV1 (human parainfluenza virus type 1) avoids MAD5 activation with the help of C protein which reduces the accumulation of dsRNA in the cytoplasm (Boonyaratanakornkit et al. 2011).

The ssRNA genome of HIV-1 undergoes reverse transcription in order to integrate few copies of its DNA into the host chromosome. However, the remaining copies could still be detected by DNA sensors such as cGAS present in the cytoplasm (D. Gao et al. 2013) (**Figure 3.5**). HIV-1 was shown to employ host encoded 3'-5' DNase - TREX1 (3'-repair exonuclease 1) to bind to these extra copies of HIV-1 DNA, initiate its degradation and thereby evade cGAS sensing (Yan et al. 2010). HIV-1 reverse transcripts were shown to be accumulated in the TREX-1 deficient host cells. This caused the activation of STING and initiated IFN production in these cells. HIV-1 spread and replication was strongly inhibited in these TREX1 deficient host cells. In addition to this, HIV-

1 DNA sensing by cGAS was shown to be blocked by HIV-1 capsid dependent recruitment of cellular protein, CYPA (cyclophillin A) (Rasaiyaah et al. 2013; Lahaye et al. 2013). However, mechanism by which CYPA inhibits cGAS activation upon HIV-1 cDNA recognition needs to be studied further and is unclear at the moment. cGAS-STING signaling was recently shown to be inhibited by HAdV-C5 (human adenovirus 5) and HPV18 (human papillomavirus 18) with the help of viral proteins E1A and E7 respectively (Lau et al. 2015). LXCXE motifs present in these viral proteins were reported to interact with STING, instead of cGAS. However, it is not known if this interaction actually affects ubiquitylation or dimerization of STING. vIRF1 (viral interferon regulatory factor 1) encoded by KSHV inhibits TBK1-dependent phosphorylation of STING by binding to it (Ma et al. 2015). This interaction between TBK1 and vIRF1 inhibits TBK1 binding and the activation of STING.

Viral RNA modification (Figure 3.4): Several viruses belonging to Bunyaviridae family, for example, CCHFV (Crimean-congo haemorrhagic virus) and Hantaan virus) and Bornaviridae family (BDV (Borna disease virus) escape RIG-I surveillance by converting 5'-triphosphate group to 5'-monophosphates in their genome with the help of virus encoded phosphatases (Habjan et al. 2008; Hao Wang et al. 2011). Junin virus, a member of Arenaviruses, contains 5' overhang in their genome and this was shown to inhibit the induction of type 1 IFNs (Marq et al. 2010). It is well known that dsRNA with a 5'-triphosphate group and an overhang functions as RNA decoy (it does not activate RIG-I but just binds to it) (Marq et al. 2011). Lassa virus employs an interesting strategy to inhibit RIG-I activation with the help of nucleoprotein which adopts a 3D structure with 3'-5' exonuclease activity and promotes the digestion of free dsRNA generated by the virus. This structure resembles exonucleases belonging to DEDD superfamily (Hastie et al. 2011; Reynard et al. 2014).

Virus mediated post translational modifications: Post-translational modification of RLRs and signalling molecules downstream to RLRs (for example, threonine/serine phosphorylation and ubiquitylation) is an important factor in the regulation of RLR signaling **(Figure 3.4)**. A lot of viruses specifically target RIG-I-Ly63-linked ubiquitylation which is required for RIG-I activation. Other viruses are known to target host E3 ubiquitin ligase which mediates RIG-I ubiquitylation. As an example, coiled-coil domain of IAV-NS1 protein prevents TRIM25 homo-oligomerization by interacting with it directly. TRIM25 oligomers facilitate the attachment of Lys63-linked ubiquitin polymers to RIG-I CARDs at Lys172 and thus are important for RIG-I activity (Gack et al. 2009). Likewise, HCV employs NS4A-NS3 protease complex to degrade Riplet and thereby blocks RIG-I ubiquitylation (Oshiumi et al. 2013). Some viruses (including DNA viruses and RNA viruses) encode DUBs (for example, L^{pro} encoded by FMDV (Foot and mouth disease virus), PLP (papain-like protease) from SARS-CoV (severe acute respiratory syndrome-associated coronavirus), OTU (ovarian tumour) type DUBs from nairoviruses and arteriviruses) and ORF64 of KSHV) which facilitates the removal of Lys63-linked ubiquitin polymers from RIG-I (Inn et al. 2011; Clementz et al. 2010; D. Wang et al. 2011; van Kasteren et al. 2013). In the non-infected cells, MDA5 and RIG-I are kept in a inactive state due to the phosphorylation of its threonine

and serine residues. However, in the presence of virus infection, recruitment of PP1γ and PP1α (PP1 phosphatases) causes the dephosphorylation of CARDs which then Initiates innate immune sensing (discussed above). Non-structural protein V of MeV (measles virus) sequesters PP1 proteins from MDA5 by binding to it at the C-terminal end (Davis et al. 2014). Similar strategy of targeting PP1 proteins was also observed in NiV (Nipah virus). An interesting thing to note was that PP1γ or PP1α caused desphosphorylation of V proteins from NiV and MeV suggesting their functionality as decoy substrates. However, MeV can also adopt protein V independent strategy to stop MDA5 and RIG-I dephosphorylation in dendritic cells. MeV interacts with CD209 (dendritic cell specific CLR protein also referred as DC-SIGN) and induces its activation which then causes the activation of RAF1 kinase. PP1γ and PP1α activity is negatively regulated as a result of this activation and thereby inhibits MDA5 and RIG-I dephosphorylation (Mesman et al. 2014). HBV polymerase was shown to block interferon β production by binding to STING (through its ribonuclease H and reverse transcriptase domains) (Liu et al. 2015) (Liu et al. 2015) (**Figure 3.5**). This interaction repressed Lys63-linked ubiquitylation of STING. PLPs (papain like proteases) encoded by several RNA viruses (SARS-CoV, PEDV (porcine epidemic diarrhoea) and HCoV-NL63 (human coronavirus NL63)) were shown to interact with STING and prevent the formation of its dimers and the attachment of Lys63-linked ubiquitin polymers (**Figure 3.5**). This subsequently inhibited IRF3 activation (Chen et al. 2014; Sun et al. 2012; Xing et al. 2013). HCV encoded, NS4B protein was also reported to interact with STING but the functional relevance of this association needs to be studied further (Nitta et al. 2013; Ding et al. 2013) (**Figure 3.5**).

RLR – MAVs degradation (Figure 3.4): Proteases encoded by several viruses facilitate RLR cleavage and this is one of most important ways by viruses can evade immune sensing. Both EV71 (enterovirus 71) and poliovirus encode $3C^{pro}$ which promotes RIGI cleavage, while, MDA5 is specifically cleaved by $2A^{pro}$ encoded by RV71 (Barral et al. 2009; Feng et al. 2014). Since MAVS is an important adaptor protein required for MDA5 and RIG-I mediated signaling, most of viruses encode for proteases which promotes MAVS cleavage. Some of the notable examples include $3C^{pro}$ and $2A^{pro}$ encoded by rhinovirus, NS4A-NS3 of HCV, $2A^{pro}$ encoded by EV71, $3C^{pro}$ of CVB3 (coxsackievirus B3) and $3C^{pro}$ encoded by HAV (hepatitis A virus) (Feng et al. 2014; Mukherjee et al. 2011; X.-D. Li et al. 2005; Yang et al. 2007; Chen et al. 2007; Drahos & Racaniello 2009). On the contrary IAV promotes non-proteolytic degradation of MAVS. IAV encodes for a small accessory protein, PB1-F2 which induces mitochondrial fragmentation and thus blocks MAVS dependent signaling (Yoshizumi et al. 2014). Additionally, PB1-F2 interacts with MAVS at its transmembrane region and inhibits the production of type 1 IFNs (Varga et al. 2012). Instead of RLR cleavage, some of the viruses also have developed strategies to reduce the abundance of MAVS and RLRs. HBV encodes for X protein that ubiquitylates MAVS at Lys136 to promote its degradation. However, the cellular enzyme used for this ubiquitylation is unknown (Wei et al. 2010). Poliovirus mediates caspase and proteasome-dependent cleavage of MDA5 rather than making use of viral proteases $3C^{pro}$ and $2A^{pro}$ (Barral et al. 2009). MeV, on the other hand induces selective autophagy (also known

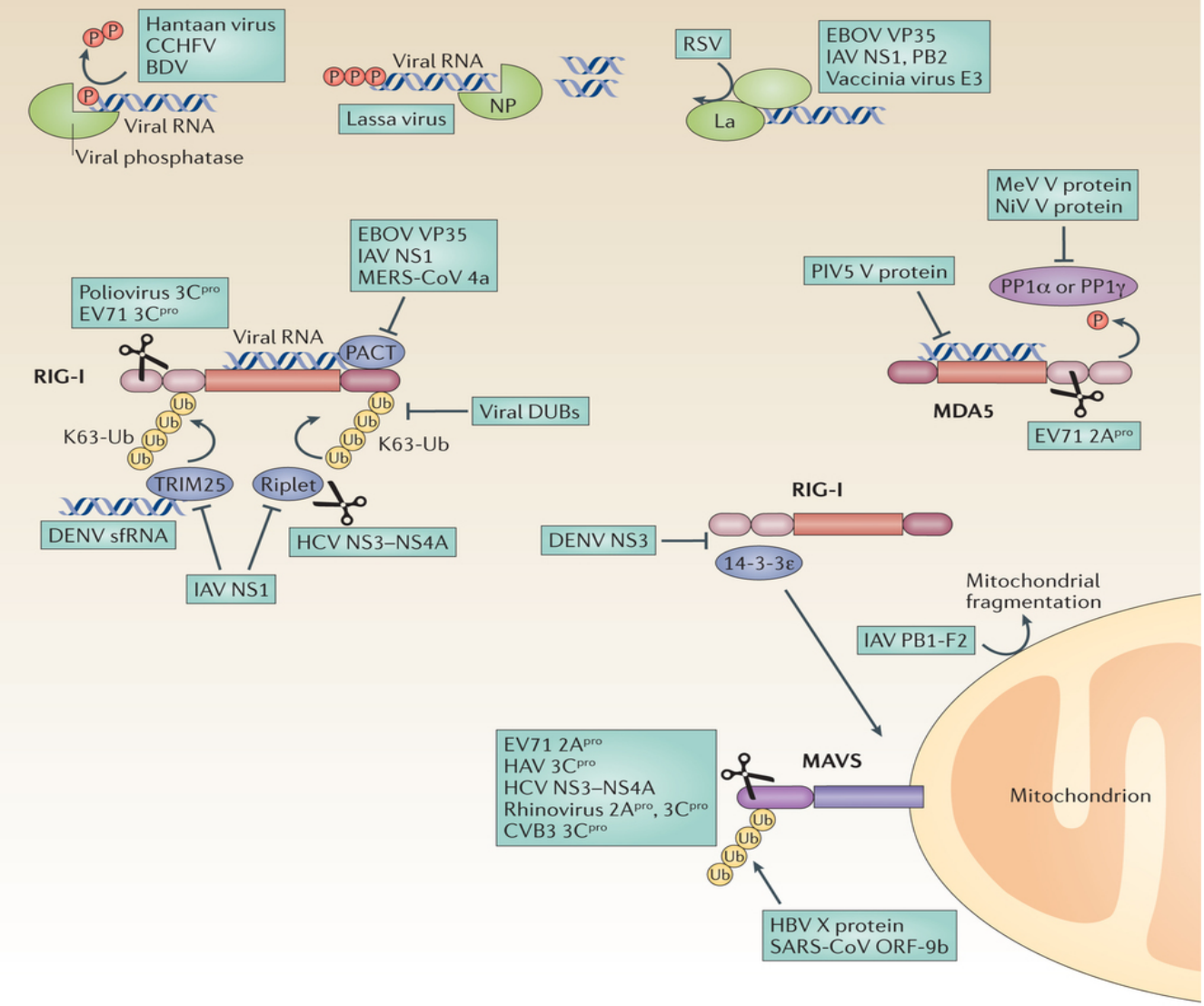

Figure 4.4 Evasion of RLR signaling by Viruses (Kai Chan & Gack 2016). Viruses have adopted several strategies to avoid or block the activation of RIG-I, MDA5 and MAVS. RIG-I activation is inhibited by virus encoded phosphatases which causes the conversion of 5'-triphosphate group to 5'-monophosphates in the viral RNA genome. Viral nucleases (Lassa virus encoded NP) can trigger the digestion of free dsRNA. Also, RIG-I recognition of viral PAMPs can be inhibited by the binding of host proteins (La) or specific viral proteins to the viral genomic RNA (For example, EBOV encoded VP35 protein and IAV encoded PB2 or NS1 protein). RIG-I and MDA5 signaling is inhibited by several viruses that regulate specific post-translational modifications of these PRRs. For instance, RIG-I signaling can be repressed by viral DUBs which facilitates the removal of Lys63-linked ubiquitin polymers from RIG-I. IAV encoded NS1 and HCV encoded NS4A-NS3 protease complex antagonize Riplet and/or TRIM25 and thus block the RIG-I activation by preventing its ubiquitylation. In addition to this, RIG-I signalling is blocked by the binding of DENV encoded sfRNA (small flaviviral RNA) to TRIM25. MDA5 is retained in its phosphorylated and inactive state by V proteins (encoded by NiV and MeV) which prevents MDA5 desphosphorylation (mediated by PP1γ or PP1). MDA5 activation is supressed by inhibiting its desphosphorylation. ATPase activity of MDA5 is blocked by PIV5 encoded V protein. Additionally, NS1 protein encoded by IAV, 4a protein encoded by MERS-CoV and VP35 protein encoded by EBOV target PACT to supress RIG-I activation. DENV encoded NS3 protein targets 14-3-3e (Mitochondrial targeting chaperone protein) to block RIG-I translocation to MAVs in mitochondria. Cleavage of MAVS, MDA5 and/or RIG-I is promoted by several virus-encoded proteases (^{pro}). IAV-encoded PB1-F2 protein is transported to the inner membrane space of mitochondria and induces mitochondrial fragmentation. Other viral proteins (HBV encoded X protein and SARS-CoV encoded 9b protein) induce MAVs ubiquitylation and its degradation. *RIG-I, retinoic acid-inducible gene-I protein; MDA5, melanoma differentiation associated protein 5; MAVS, mitochondrial antiviral signaling*

protein; NPs, nucleoproteins; PAMPs, pathogen associated molecular patterns; La - RNA binding protein; EBOV, Ebola virus; VP35, viral protein 35; IAV, influenza A virus; PB2, Polymerase basic protein 2; NS1, non-structural protein-1; TRIM25, tripartite motif protein 25; HCV, hepatitis C virus; DENV, dengue virus; sfRNA, subgenomic flavivirus RNA; NiV, Nipah virus; MeV, measles virus; PP1, protein phosphatase 1; PIV5, parainfluenza virus 5; MERS-CoV, middle east respiratory syndrome coronavirus; EBOV, ebola virus; PACT, protein kinase R activator; HBV, hepatitis B virus; SARS-CoV, severe acute respiratory syndrome-associated coronavirus.

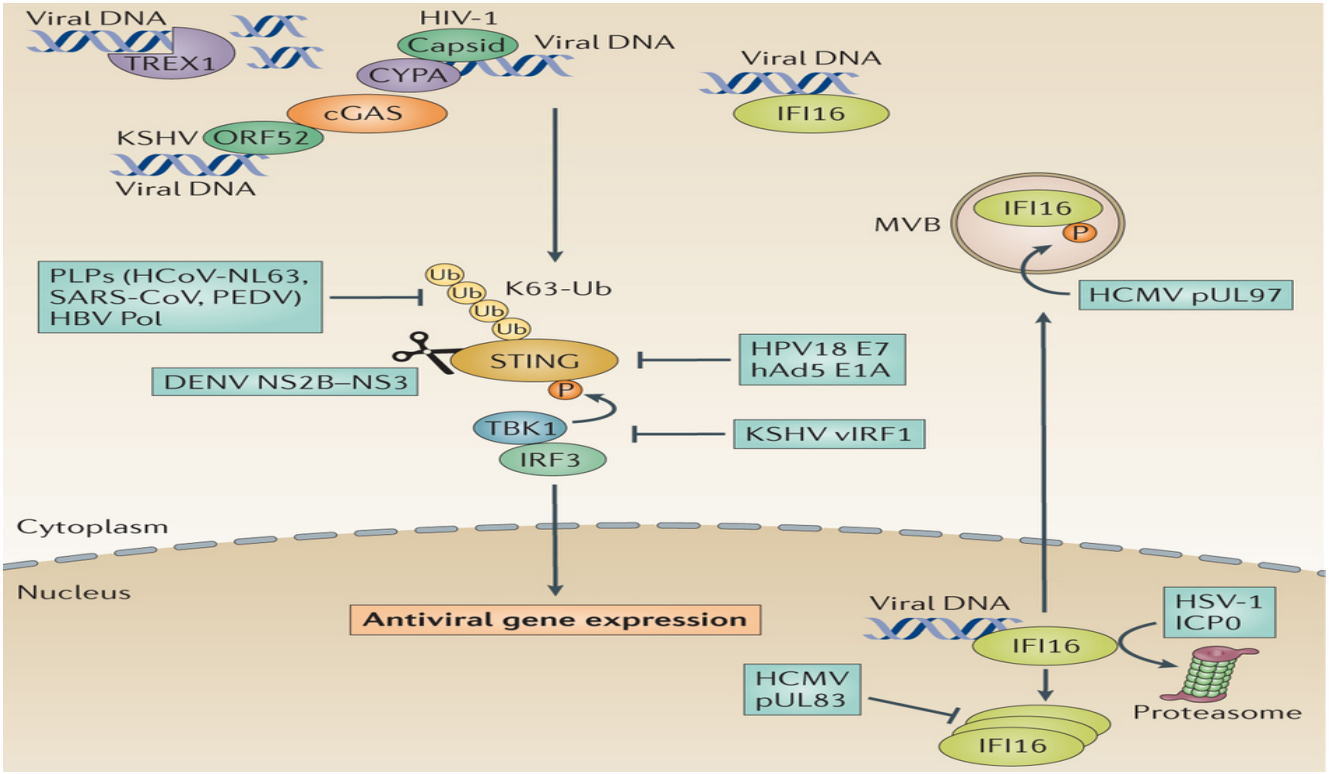

Figure 4.5 Evasion of IFI16 and cGAS signaling by Viruses (Kai Chan & Gack 2016). Several strategies are adopted by DNA viruses to avoid or block the activation of intracellular DNA sensors. HIV-1 makes use of cellular protein TREX1 to facilitate the degradation of extra copies of reverse transcribed viral DNA. Host factors such as CYPA are recruited by HIV-1 capsids to block cGAS sensing of reverse transcribed viral DNA. Additionally, KSHV encoded ORF52, (tegument protein) binds to cGAS and as well as viral DNA and inhibits cGAS enzymatic activity. STING activation is antagonized (by the removal of Lys63-linked ubiquitin polymers from STING or preventing the attachment of ubiquitin polymers to the STING) by HBV encoded polymerase and PLPs encoded by several viruses (SARS-CoV, HCoV-NL63 and PEDV). vIRF1 encoded by KSHV inhibits TBK1-dependent phosphorylation of STING and thereby blocks its signaling. E7 protein encoded by HPV18 and E1A protein encoded by HAdV-C5 inhibit STING activation by binding to it, whereas, STING is cleaved by DENV encoded NS3-NS2B protease. HSV-1 encoded ICP0 inhibits IFI16 in the nucleus by targeting it for proteasomal degradation. On the other hand, HCMV encoded pUL83 (tegument protein) binds to IFI16 and prevents IFI16 oligomerization and thereby its signaling. IFI16 is phosphorylated into an inactive state by HCMV encoded pUL97 which causes IFI16 relocalization to MVBs from nucleus. *IFI16, IFNγ inducible protein 16; cGAS, cyclic GMP-AMP synthase; HIV-1, human immunodeficiency virus-1; TREX1, 3' repair exonuclease 1; CYPA, cyclophillin A; KSHV, Kaposi sarcoma-associated herpesvirus; STING, stimulator of IFN genes; HBV, hepatitis B virus; PLPs, papain like proteases; SARS-CoV, severe acute respiratory syndrome-associated coronavirus; HCoV-NL63, human coronavirus NL63; PEDV, porcine epidemic diarrhoea; vIRF1, viral interferon regulatory factor 1; TBK1, tank binding kinase 1; HPV18, human papillomavirus 18; HAdV-C5, human adenovirus 5; DENV, dengue virus; HSV-1, herpes simplex virus 1; ICP0, viral E3 ubiquitin ligase infected cell protein 0; HCMV, human cytomegalovirus; MVBs, multivesicular bodies.*

as mitophagy) and promotes the degradation of mitochondria which significantly reduces abundance of MAVS (Xia et al. 2014). RSV promotes proteasome-dependent cleavage of RIG-I and other immune molecules in mitochondria, except MAVS by the formation of large degradative complex with the help of NS1 and NS2 proteins (Goswami et al. 2013).

IFI16 and STING degradation (Figure 3.5): HSV-1 replicates in the nucleus. It was shown to promote proteasome dependent IFI16 degradation (Orzalli et al. 2012). Authors in this study claimed that IFI16 degradation was mediated by ICP0 (viral E3 ubiquitin ligase infected cell protein 0) based on the results obtained from the mutant studies. IFI16 is not degraded in Mutant HSV-1 lacking functionally active ICP0. It was shown that IFI16 interacts with ICP0 and this caused the recruitment of ICP0 foci to the nucleus before degradation (Orzalli et al. 2012). In case of STING, DENV encoded NS3-NS2B complex facilitates the degradation of STING which blocks IFN induction (Yu et al. 2012; Aguirre et al. 2012). However, NS3-NS2B is only able to cleave Human STING but not mouse STING as they did not contain any cleavage sites. This implies that the restriction imposed by STING on DENV replication is species specific. Thus, proteases encoded by viruses such as DENV and HCV (NS4A-NS3) (shown above) can have a major impact on adaptor proteins of RNA and DNA sensors.

RLR relocalization or sequestration (Figure 3.4) : PIV5 (parainfluenza virus 5) encodes the V protein which sequesters MDA5 and retains it in an inactive state by binding to its helicase domain (Andrejeva et al. 2004; Rodriguez & Horvath 2013; Motz et al. 2013; Childs et al. 2009). This causes the inhibition of MDA5-ATPase activity. Several viruses encode specific proteins, for example, IAV-NS1 protein, MERS-CoV (middle east respiratory syndrome coronavirus)-4a protein and EBOV-VP35 protein which targets PACT and this suppresses RIG-I and MAD5 activation (Luthra et al. 2013; Tawaratsumida et al. 2014; Siu et al. 2014). Additionally, RIG-I is sequestered from RLR signaling pathway and its downstream molecules by binding to several other viral protein, for example, hMPV (human metapneumovirus)- glycoprotein, arenavirus-Z protein, SARS-CoV-membrane protein (Siu et al. 2009; Fan et al. 2010; Xing et al. 2015; Bao et al. 2008). Upon MDA5 and RIG-I recognition of non-self-cytosolic RNA, MDA5 and RIG-I needs to be transported to specific organelles that abundantly expresses MAVS (mitochondria, peroxisomes and MAMs). Recent study reported that DENV encoded NS3 protein binds to 14-3-3e (Mitochondrial targeting chaperone protein) to inhibit RIG-I translocation to mitochondria and thereby blocking the activation of MAVS (Chan & Gack 2016). On the contrary, HCV encoded NS4A-NS3 phosphatase complex interestingly promotes the proteolysis independent cleavage of MAVS to inhibit RIG-I signaling (X.-D. Li et al. 2005)(discussed above). Virus can also promote relocalization of RLRs to specific sites within the cell or to virus induced structures (For example, Inclusion bodies and endosome like structures). RSV encoded nucleoprotein interacts with MDA5 and facilitates its relocalization (along with MAVS) to virus induced large inclusion bodies (Lifland et al. 2012). On the other hand

SFTSV (severe fever with thrombocytopenia syndrome virus) encoded small segment non-structural proteins promotes relocalization of RIG-I and TRIM25 (upstream activator of RIG-I) into virus induced endosome like structures in the cytoplasm (Santiago et al. 2014).

DNA sensor relocalization or sequestration (**Figure 3.5**): KSFV encoded tegument protein, ORF52 was recently reported to inhibit cGAS signaling (Wu et al. 2015). ORF52 binds to cGAS and as well as viral DNA. This binding caused the repression of cGAS enzymatic activity and subsequently supressed cGAMP production. Similarly, ORF2 homologues found in other gammaherpesviruses, for example, MHV68 (murine gamma herpesvirus 68) and EBV also inhibited the cGAS activity. This implied conserved function of ORF52 in the evasion of innate immune sensing. In comparison to HSV-1, HCMV adopts different mechanism to target IFI16. Although, HCMV DNA is sensed by IFI16, the tegument protein pUL83, encoded by the virus binds to IFI16 at its pyrin domain and antagonizes immune signaling by preventing the formation of IFI16 oligomers (T. Li et al. 2013). Interaction between IFI16 and pUL83 occurs in a concerted manner where N-terminal end of pUL83 first binds to IFI16. IFI16 oligomerization is then dissipated by C-terminal end of pUL83. Additionally, it was found that phosphorylation of pUL83 at Ser364 significantly reduced its efficiency to antagonize IFI16. This implied that immunosuppressive activity of pUL83 can also be regulated by host kinases. Another complementary strategy employed by HCMV to antagonize IFI16 activation has been reported recently (Dell'Oste et al. 2014). At the time of HCMV replication, IFI16 is mislocalized to MVBs (multivesicular bodies) during its translocation to cytoplasm from the nucleus. Thus, HCMV DNA is prevented from being recognized in the nucleus. In MVBs, IFI16 is phosphorylated by viral kinase, pUL97 and is shown to be important for the egress of IFI16 from the nucleus.

Chapter 2

Second chapter of this thesis gives details about the first project carried out during my PhD. This work was a collaborative effort of several people (listed below) with me being the main contributor, particularly figure 1A, 1D, 2A, 2B, 3A, 3B, 4C and all the supplementary information. The main aim of the project was to identify cellular miRNAs differentially regulated by human adenoviruses and to study their role in infection. This project mainly focuses on host miRNAs, particularly, miR-29b-1* and miR-27b*. These miRNAs were significantly downregulated upon infection. The experiments carried out in this project included miRNA expression profiling by microarray, *in-silico* approaches to predict the host and viral targets of miR-29b-1* and miR-27b*, transient transfections, infection assays and host transcriptome profiling by whole genome microarrays. This chapter is divided into following sections: 1) Abstract, 2) Introduction to the topic, 3) Results derived from the experiments, 4) Discussion of the results and importance of this work, and 5) Materials and methods used in this project.

Chapter 2

Characterization of cellular miRNAs regulated by human adenoviruses and their impact on host factors by systems profiling

(Manuscript in preparation)

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Abstract

Adenoviruses are non-enveloped DNA viruses infecting many different vertebrates, including humans. Depending on the serotypes causing infection, adenoviruses are responsible for clinical diseases like gastroentitis, conjunctivitis, pneumonia, myocarditis and hepatitis. For immune-compromised individuals, adenoviruses are a major risk factor, and infection can lead to severe health problems and fatalities. In this work, we studied the expression profiles of host microRNAs (miRNAs) in the course of human adenovirus (HAdV-C5 and HAdV-B3) infection of human A549 cells using custom made Agilent Microarrays. Several cellular host miRNAs were found to be differentially regulated upon HAdV-C5 or HAdV-B3 infections. However, four miRNAs (hsa-miR-21-3p, hsa-miR-29b-1*, hsa-miR-27b* and hsa-miR-125a-5p) were strongly downregulated ($Log_2FC < -0.5$ and $FDR < 0.05$) and two miRNAs (hsa-miR-1202 and hsa-miR-1273g-3p) were strongly upregulated ($Log_2FC > 0.5$ and $FDR < 0.05$) in both the infections. Additionally, we carried out profiling of cellular genes by genome microarrays in A549 cells transfected with miR-29b-1* and miR-27b* Qiagen mimics to obtain a list of potential genes induced or suppressed by these miRNAs. We observed significant upregulation of interferon-regulated genes and downregulation of cell cycle and cell adhesion genes. In addition, we also found that miR-29b-1* and miR-27b* could target some of the important HAdV genes and some of the host factors involved in the virus entry based on the miRNA-mRNA sequence complementarity assessed through bioinformatic approaches. These studies were carried out to identify relevant host or viral mRNA targets and to reveal new properties of host and viral regulatory networks regulated by these differentially regulated miRNAs.

Introduction

Adenoviruses are non-enveloped, icosahedral DNA viruses with a genome size of 26-45 kbp, capable of infecting many different vertebrates, including humans (Berk 2007; Fields et al. 2007). There are more than 75 serotypes of human adenoviruses (documented in Genbank) and these viruses can be categorized into seven species (A-G) based on biochemical and sequence analysis (Benkö & Harrach 2003; Echavarría 2008). Among human adenoviruses (HAdV), species C human adenoviruses are the best studied viruses (Fauquet et al. 2005). Considerable amount of research has been directed towards the understanding of the virus-host interactions. Systems analyses of virus host interactions typically involve high-throughput molecular profiling and computational analyses, often with an overall aim to pave the way for new antiviral therapies and diagnostics as well as vaccinology approaches (Law et al. 2013; Pulendran et al. 2010). For example, recent system-wide analyses in SARS coronavirus or influenza virus infected cells have shown a differential expression of RNAs not encoding proteins, including long (larger than 200 bases) noncoding RNAs, small nuclear RNAs (snoRNAs) or piwi-associated small RNAs (piRNA) (Mercer et al. 2009; Peng et al. 2010). Likewise, RNA-sequencing showed that HIV infection of a model T cell line led to differential regulation of miRNA host genes (Chang et al. 2011). It was also intriguing to note that the profiles of noncoding RNA were often similar in virus infected cells, and cells stimulated with type 1 interferon (IFN), implicating that noncoding RNAs may be part of regulatory mechanisms involving innate immune response of the cells against viruses, at least in cell culture systems. Such data are increasingly enhancing the concept of the host transcriptome, and are manifested for example in the Encyclopedia of DNA Elements (ENCODE) project (Consortium et al. 2012). With this regard, we wanted to study the systems properties of Human adenovirus (HAdV-C5 and HAdV-B3) infected cells with respect to host miRNAs.

MicroRNAs (miRNAs) are small non-coding RNAs with key regulatory functions for eukaryotic transcripts, in particular their stability (Bartel 2004). Although viruses frequently encode their own miRNAs (Kozomara & Griffiths-Jones 2014), they also control the expression of host miRNAs and thereby alter pathways and regulatory networks of the cell for their benefit (Guo & Steitz 2014). Immune response staged against microbial infections could be one of the ways by which host miRNAs may offer protection against bacteria, viruses, fungi and other parasites. RNAi mediated inhibition of viral replication facilitated by exogenous siRNA or miRNA in the host cells is still a matter of debate (Bogerd et al. 2014). Currently there are no specific antiviral agents available for treating adenoviral

infection, most of the treatment is focussed on the symptoms. Interference of viruses with each other during its growth and replication in the host cells often leads to the regulation of innate immune response, resulting in the production of Interferon (IFN). Many studies have highlighted the prominence of IFN in the treatment of several infectious diseases such as hepatitis C (Sharieff et al. 2002). Several studies have also reported the importance of RNA interference (RNAi) as strategy to combat infections in-vitro, such as Coxsackie virus (A16) and Coxsackie virus B3(Wu et al. 2008; Wu et al. 2005; Yuan et al. 2005). Although majority of human viruses during the acute stages of infection and replication are quite resistant to endogenous small RNA or miRNA mediated restriction, there are some strong evidences to suggest that miRNAs may be involved in inhibiting or boosting the infection and replication of some viruses like Hepatitis virus B (HBV) and C (HCV) (Jopling et al. 2005; Kitab et al. 2015). Majority of the miRNAs encoded by the host cells inhibit viral replication either by directly targeting the viral genome or indirectly by regulating a host factor associated with replication (Shrivastava et al. 2015). In addition to HCV and HBV, the role of host miRNAs in regulating infection caused by several viruses such as dengue virus (DENV) (S. Wu et al., 2013;Castillo et al., 2016;Wen et al., 2015), herpesviruses (Frappier, 2015; F. Lu et al., 2008; Gatto et al., 2008) and human Immunodeficiency virus-1 (HIV-1) (Kakumani et al. 2013) has been well studied.

In this study, we carried out the profiling of host microRNAs (miRNAs) in the course of human adenovirus (HAdV-C5 and HAdV-B3) infection of human epithelial lung A549 cells. We found that few miRNAs were upregulated in HAdV infected cells, suggesting that they might enhance infection. miRNAs could be also upregulated as a part of host antiviral response or upregulation could be just collateral damage upon infection. We also found several downregulated host miRNAs in these infected cells. We validated these results with the help of quantitative reverse transcription polymerase chain reaction (qRT-PCR) and found that two host miRNAs - miR-29b-1* and miR-27b* were significantly downregulated, 24h and 48 post infection (p.i). Later we performed transcriptome analyses of cells transfected with the commercially available mimics of these miRNAs to identify potential host target genes differentially regulated by these miRNAs like molecules and to understand their role in adenovirus infection dynamics. Since the seed region of the miRNA (region which extends from 2nd nucleotide to the 8th nucleotide from the 5' end) is known to interact with the target mRNA most of the times, we sought with a bioinformatic approach to predict adenoviral gene targets based on the sequence complementarity between the miRNA seed and mRNA. However, in few cases miRNAs are also known to interact with the target mRNA in a non-seed dependent manner. With this regard, we found that some of the key HAdV and host genes could be targeted by

miR-29b-1* and miR-27b*.

Results

Several Host miRNAs are differentially regulated in HAdV-C5 and HAdV-B3 infected cells

In order to understand the role of human host miRNAs during adenovirus infection, we performed miRNA expression profiling in adenovirus infected A549 cells using custom made Agilent Microarrays. A549 cells were mock-infected (growth medium without virus) or infected with HAdV-C5 and HAdV-B3 and the total RNA was extracted 24h and 48h post infection (p.i.). The quality of RNA was assessed by measuring the RNA integrity number (RIN) which was confirmed to be above 7 in all the samples. Expression of differentially regulated miRNAs during HAdV-C5 and HAdV-B3 infection is depicted in a Heatmap (**Figure 1A**). Fold changes and Adjusted P values (False Discovery rate[FDR]) of the differentially regulated miRNAs ($Log_2FC > 0.5$ or $Log_2FC < -0.5$ at 24hpi or 48hpi and FDR < 0.05) are summarized in **Table S1 and Table S2**. We observed that maximum amount (71%) of differentially regulated miRNAs (FDR < 0.05) were downregulated in HAdV-C5 infected cells at 24h p.i. Additionally, we noticed upregulation of significant amount of differentially expressed miRNAs at 48h p.i. compared to 24h p.i. (29% to 44%). Furthermore, upon HAdV-B3 infection, we observed a significant upregulation of differentially expressed miRNAs (62% at 24h p.i. and 67% at 48h p.i.) relative to mock-infected control (**Figure 1B**). Only four miRNAs (hsa-miR-21-3p, hsa-miR-29b-1*, hsa-miR-27b* and hsa-miR-125a-5p) were found to be strongly downregulated ($Log_2FC < -0.5$ and FDR < 0.05) in both HAdV-C5 and HAdV-B3 infected cells at both time points **(Figure 1C** (top panel)**, Table S1)**. Two strongly upregulated ($Log_2FC > 0.5$ and FDR < 0.05) miRNAs (hsa-miR-1202 and hsa-miR-1273g-3p) were found to be common among each time point and virus infections **(Figure 1C** (bottom panel)**, Table S2)**. Comparative qRT-PCR analysis was carried out to validate the results obtained from microarray data. miR-29b-1*, miR-27b*, miR-21-3p and miR-125a-5p were selected for validation, in particular as they were significantly downregulated at 24h and 48h post HAdV-C5 and HAdV-B3 infections. Using miRNA q-RT-PCR, we determined the relative fold change of multiple miRNAs over the course of infection **(Figure 1D)**. The values in the graph represents the mean absolute fold change of triplicate experiments for each miRNA at each individual time point compared to mock-infected controls collected at each time point. Expression levels of the selected miRNAs in the infected cells were normalized to the levels in the mock-infected controls. q-RT-PCR data confirmed the downregulation of two miRNAs (has-miR-29b1* and hsa-miR-27b*) and these were chosen for further analyses.

miR-29b-1* and miR-27b* could target key host and adenoviral genes

miR-29b-1* and miR-27b* miRNAs were predicted to interact with host factors like CXADR (Coxsackievirus and Adenovirus Receptor) and ITGAV (Integrin alpha-v) mRNAs either in a seed dependent manner or via non-canonical interaction (sequences outside the seed region were found to be interacting with the mRNA targets) (**Figure 2A**). The binding energies of the miRNA-mRNA interactions were estimated and scored by MIRZA model (Khorshid et al. 2013). Additionally, bioinformatics analyses also suggested that the seed sequences of both these miRNAs could directly target some of the HAdV-C5 genes, including E1B, pIX, E3, Iva2 and pol based on the miRNA seed sequence complementarity with 3'UTR region of these genes (**Figure 2B**).

Synthetic miRNA mimics of host miR27b* and miR29b-1* inhibit HAdV infection of A549 and WI38 cells

We tested the effects of all the downregulated miRNAs in the form of synthetic miRNA mimics from Qiagen on HAdV infection. Synthetic miR-29b-1*, miR-27b*, miR-21-3p and miR-125a-5p along with negative control siRNA (siAllstar) were transfected in A549 and WI38 (human embryonic lung fibroblast) cells for 24h and cells were infected with wild type HAdV-C5 and analyzed 24 hrs post-infection (p.i.) The details of the mimics are given in **supplementary table S8.** The infection dose used gave about 50% infected cells in the negative (non-transfected) control. After infection, the cells were fixed and immunostained for the late viral protein VI. miR27b* and miR29b-1* were the most effective at reducing HAdV infection, notably with some loss in cell numbers (**Figure 3**).

miR27b* and miR29b-1* mimics induce a vigorous type I interferon response

To explore the mode of action of the antiviral Qiagen miRNA mimics, we performed gene-expression profiling of the mimic-transfected A549 cells using whole genome microarrays from Agilent technologies. A549 cells were transfected with blunt ended miRNA mimics of miR-29b-1*, miR-27b* and non-targeting siRNA (siAllstar) control. The extracted RNAs were labelled with Cy3 and hybridized to custom-made whole genome microarrays. Considering the overrepresentation of miR-27b* and miR-29b-1* upon mimic transfection, we expected to find mostly downregulated genes among the 19417 genes tested. Surprisingly, we not only found a rather high number of differentially up-regulated genes, but also that the genes were strongly up-regulated compared to the control with a differential expression of up to 11 log2-folds. The up-regulated gene list included several Interferon response genes. **Table S3 and Table S4** gives an overview of common top 50 most differentially up

and downregulated genes for each miRNA mimic. Entire gene lists with log_2FC and p values are provided in **Supplementary file 2.** We identified 7617 transcripts that exhibited statistically significant changes in expression upon blunt-ended and non-modified miR29b-1* and miR27b* mimic transfection with FDR values < 0.05. This represents about 38% of the total interrogated host transcripts in the microarray **(Figure 4A)**. More than 65% of the differentially up-regulated genes (1894 out of 2596) with $log_2FC \geq 1$ and down-regulated genes (1754 out of 2652) with $log_2FC \leq -1$ were largely identical in both miRNA mimic transfected cells (**figure 4B).** To further classify these differentially regulated genes into gene sets and to identify potential biological pathways, we carried out enrichment analysis of these gene lists. We applied the differentially expressed gene lists, with their log_2FCs and p values to the MetaCore system (Ekins et al. 2007) and performed bioinformatics pathway enrichment analysis on these data. This revealed 7 lists of significant-scoring pathway maps (**Supplementary table S5 and table S6**). Most of the upregulated genes from the pathway enrichment turned out to be innate immune response and interferon–induced genes like MX1, MX2, IFIT2/3 (interferon-induced protein with tetratricopeptide repeats 2/3), OAS2 (2'-5'- oligoadenylate synthase2), IFNB1 (Interferon Beta), STAT1 or IRFs. The GO processes for the downregulated genes were associated with cell adhesion and cell cycle (**Supplementary table S6 and Figure 4C**). This suggested that the transfected miR-29b-1* and miR-27b* mimics could lead to induction of type I interferon response, and perhaps thus explain the antiviral effect of these mimics.

Discussion

miRNAs are increasingly found to be regulators of cell function. It is estimated that more than one third of the human genes are regulated by miRNAs (Law et al. 2013). These findings suggest that miRNAs play an integral role in genome-wide regulation of gene expression. Considerable amount of information has been acquired from systems analyses of adenovirus infection, including expression profiling of cellular and viral genes upon miRNA transfection and HAdV5 infection(Granberg et al. 2005; Granberg et al. 2006; Zhao et al. 2009) , host and viral protein expression profiling upon HAdV-C5 infection (Trinh et al. 2013), as well as miRNAs (Bellutti et al. 2015). It is feasible to make efforts towards integrating the data and arriving at list of genes or proteins that are commonly regulated in one particular type of cell. For example, it was found that adenoviruses can suppress RNAi machinery at the latter stages of infection with the help of virus-encoded VA-RNAs and thereby causing general reduction of host miRNA levels (Andersson et al. 2005). However, in addition to downregulated miRNAs some of the host miRNAs were found to be upregulated upon HAdV-B3 infection of human

laryngeal epithelial cells for 72h (Qi et al. 2010). Additionally, our results from the miRNA expression profiling experiment performed on HAdV-C5 and HAdV-B3 infected A549 cells, showed several differentially regulated (both up and down-regulated) host miRNAs, 24h and 48h p.i. Interestingly, in these miRNA profiles were significant downregulated star miRNAs (miR-27b* and miR-29b-1*) in comparison to the abundantly expressed normal guide strands (miR-27b and miR-29b-1). This finding was validated and further confirmed by qRT-PCR analysis of downregulated miRNAs under the same infection conditions. miR-29b-1* and miR-27b* are minor miRNAs in A549 cells (Bellutti et al. 2015), and are thus not likely to have biological impact on virus infection. However, transfection of synthetic versions of either of these miRNAs in the form of mimics significantly inhibited HAdV-C5 infection of cultured cells.

The downregulation of miR-29b-1* and miR-27b* was in accordance to the previous RNA sequencing study, which highlighted these miRNAs in addition to the much longer list of downregulated cellular miRNAs upon HAdV-C5 infection (30h p.i.) in A549 cells (Bellutti et al. 2015). Notable differences between our studies and RNA sequencing studies by Bellutti et al. 2015 was the moderate amounts of HAdV-C5 particles used for the infections (10 MOI) in the latter study. In addition, reads were mapped to unique miRNA sequences from miRBase 19, hg19 (sequences from human genome stored in NCBI GenBank) and Rfam11.0. Compared to our microarray platform which made use of miRNA sequences from the previous release – miRBase 18, miRBase 19 contained 515 novel mature miRNA sequences. Interesting thing to note in the study by Bellutti et al. 2015, was the lower abundance of miR-29b-1* and miR-27b* in the non-infected cells. On the contrary, the study by Zhao et al. 2015 which studied the miRNA profiling of adeno-infected human primary lung fibroblasts (6h , 12h, 24 and 36h p.i.), did not report the downregulation of miR-29b-1* or miR-27b* upon infection. These comparisons raise the issue of reliability and reproducibility of miRNA profiling studies across different platforms. However, these differences in the miRNA expression profiles obtained from the above studies could be attributed to cell specific differences in the miRNA expression levels.

The precursor form of mir-27b maturates to miR-27b and miR-27b*. Similarly, miR-29b-1* and miR-29b are formed upon maturation of mir-29b (precursor form). In this case both the star miRNAs (miR*) are generated from 5’ arm of the respective precursor hairpin. Generally, miR-29b-1* and miR-27b* are considered to be the minor products (mostly less abundant compared to the miR-29b and miR-27b levels respectively). The finding that the virus downregulates the low levels of miR-29b-1* and miR-27b* is intriguing. We assume that the reduction in these miRNA levels could also be due

to the collateral damage inflicted upon infection. Most of the times these miR* are considered to be non-functional as a result of their low abundance. However, the expression levels of the miR* and non-star forms can vary between different cells, species and stages (Griffiths-Jones et al. 2011). Additionally, several studies have reported a prominent role of miR* in regulating host target network (Packer et al. 2008; Yoo et al. 2009; Kim et al. 2008; Tsang & Kwok 2009; Ro et al. 2007; Yang et al. 2011). Some of the miR* were reported to bind to different Argonaute proteins (Czech & Hannon 2011).

Gain-of-function studies with miRNAs can be carried out in different ways including transient transfections with miRNA mimics (Jin et al. 2015). Apart from acting as functional equivalents to miRNA guide strand, these mimics when delivered into the cells bypass maturation and processing steps of endogenous miRNA biogenesis and instantly increase abundance of quasi-mature miRNA (Søkilde et al. 2015; Jin et al. 2015). Additionally, miRNA levels can also be manipulated using other strategies. miRNAs can be overexpressed in the cell culture using vectors expressing short miRNA hairpin structures or miRNA gene itself. Since both the guide and passenger miRNA strands are expressed and are fully functional by this approach, the result of miRNA overexpression would be the combined effect of non-star and star miRNAs (Søkilde et al. 2015). This problem can be overcome by using specific miRNA mimics, where only one of the strands are kept active (For example, guide strand/non-star miRNAs or passenger strand/miR*) and the opposite strand is modified or kept inactive in profiling experiments. miRNA mimics can be efficiently delivered into the cultured mammalian cells via transient transfection and is considered to be an easy, fast and economical way to understand the functional relevance of the endogenous miRNAs. With this regard, we performed transcriptome analysis of A549 cells with commercially synthesized mimics of miR-29b-1* and miR-27b* to identify host genes differentially regulated by these miRNAs and to understand the functional importance of miR-29b-1* and miR-27b* in HAdV infection. It was reported that transiently transfected mammalian miRNA mimics mainly bring about the reduction of target mRNA levels instead of repressing its translation efficiency (Guo et al. 2010). But this may not always be true, since the study by Larsson & Nadon 2013 which re-analyzed the genome wide data from the above study by Guo et al. 2010, suggested that repression of the translation took place before miRNA mediated mRNA degradation. Similarly, ribosome profiling studies on zebrafish showed that repression of translation took place prior to mRNA degradation (Bazzini et al. 2012).

Based on the miRNA studies in zebrafish, mRNA degradation before its translational repression may not be the way by which mammalian miRNAs function under physiological conditions. Different mechanisms govern different miRNA-mRNA target interaction. Nonetheless, the mechanism by the miRNA supresses its target genes is largely dependent on type of cell expressing the miRNA, abundance of miRNA and its target genes in a particular cell type and the varying contributions from mRNA degradation and translation inhibition. The results from the gene expression profiling in A549 cells showed that transfected miR-29b-1* and miR-27b* mimics induced a vigorous type I interferon response, as indicated by the upregulation of multiple innate immune response and interferon–induced genes. Additionally, genes associated with Cell adhesion and cell cycle were strongly downregulated. Thus, expression of these miRNA mimics induces a complex response that could be composed on one hand of an innate/interferon response likely to be a major factor inhibiting virus infection, and on the other hand of significantly down-regulated genes possibly induced through specific miRNA-target interactions.

Bioinformatic analysis based on miRNA-mRNA sequence complementarity and binding energies predicted from miRNA-target interactions indicated that miR-29b-1* and miR-27b* could directly target key viral genes and some of the host factors involved in the virus entry. For this, we first considered the canonical seed based viral targets and secondarily non-canonical targets of miRNA in viral transcripts, as described in (Khorshid et al. 2013). Notably, the number of non-canonical interactions of miRNAs with mRNAs has been observed to significantly increase if the expression of miRNA was high. This emphasizes the importance of determining the absolute levels of miRNAs in the steady state of the uninfected cells. Due to the anti-adenoviral activity of miR-29b-1* and miR-27b*, it would certainly be advantageous for the virus to downregulate these miRNAs. IFN/cytokines production could be repressed by the downregulation of these miRNAs and this could have facilitated the spreading of the virus without being recognized by the immune system. However, this speculation was based on the fact that the mimics functioned as true endogenous miRNAs. In either case, the upregulation of interferon-regulated genes and several innate immune response genes by miR-29b-1* and miR-27b* mimics could be the main reason for antiviral effect of these mimics. Reduction in miRNA levels could also be caused by the host cell. For example, miRNAs can be packaged into membrane vesicles and get released to the extracellular compartments thereby alerting the neighbouring cells (reviewed by Turchinovich et al. 2016). Such a mechanism would be similar to role of secreted proteins such as IFNs that confers protection to the neighbouring cells from

viral infections and prevents the spreading of viruses in tissues. Based on results obtained from the infection assay, an interesting thing to note was the reduced cell numbers in miR-29b-1* and miR-27b* mimic transfected cells. The downregulation of cell adhesion and cell cycle genes could perhaps explain the observed drop in cell numbers in these mimic transfected cells. The downregulated genes could also be due to the off-target effects of 29b-1* and miR-27b* mimics or due to non-canonical interactions of these miRNA mimics with the target mRNAs. For example, miR-29b-1* and miR-27b* does not seem to target COL21A1 (cell adhesion gene) based on sequence complementarity between the miRNA seed and 3'UTR of COL21A1. However, COL21A1 contains a 7mer binding sites in the seed region of both miR-29b and miR-27b. Additionally it is also possible that the complementary strand of miR-29b-1* and miR-27b* mimics could be functionally active. Thus, the antiviral effect of these mimics could either be due to the on-target activity of miRNA of interest or due to the activity of its complementary strands. However, it is still unclear whether the strand complementary to miR-29b-1* for example, with small sequence dissimilarities in the seed region could still function as miR-29b (non-star for miR-29b-1*). Common Induction of interferon response by miR-29b-1* and miR-27b* mimics could indirectly explain the downregulation of cell adhesion genes. It was previously shown that upon induction of IFN response, different collagen genes were significantly downregulated (Woeckel et al. 2012). However, it is difficult to predict if the antiviral effect of these mimics is due to the reduced expression of collagen genes or due to the combined effect of all other downregulated genes and this requires further investigation. In the past, few miRNAs were reported to have broad spectrum antiviral activity against herpesvirus infections (Santhakumar et al. 2010). Recently, broad-spectrum antiviral activity of several miRNA mimics was reported in the inhibition of respiratory virus infections (McCaskill et al. 2017). The screening experiments performed in this study demonstrated the significant inhibition of several strains of influenza and RSV by miR-124, miR-24 and miR-744. This antiviral activity was attributed towards the targeting of p38 mitogen-activated protein kinase 2 (MAPK2) pathway by these miRNAs. Interestingly, the suppression of MAPK2 also gave rise to antiviral response during adenovirus infection (Suomalainen et al. 2001). However, the genes belonging to MAPK2 pathway were not found to be downregulated upon miR-29b-1* and miR-27b* mimic transfection of A549 cells.

The induction of innate immune and IFN response genes by miR-29b-1* and miR-27b* mimics could also be due to the indirect effect of these mimics. These mimics could represent PAMPs (pathogen associated molecular patterns) and be recognized by dsRNA sensors such as MDA5, RIG-I or PKR in the cell as a part of innate immune sensing. Since these mimics represent very short dsRNA molecule

(less than 25nt in length), Such small RNA molecules represent non-optimal ligands and are less likely to be recognized by the RNA sensors. Additionally, during the processing of mature miRNAs, long dsRNA intermediates are formed with imperfect base pairing. However, it is unclear if the RNA sensors could distinguish between these structures and miRNA mimics where both of its strands are completely complementary to each other. On the contrary, RIG-I has been reported to sense short dsRNAs (< 25nt) even in the absence of 5'-triphosphate groups or overhangs at the end (Marques et al. 2006; Takahasi et al. 2008; Judge et al. 2005) . However, interferon response genes were not induced by the transfection of short double stranded control siRNA – siAllstar. This presented a strong argument against the sensing by dsRNA sensors. Additionally, out of the four downregulated miRNAs, only miR-29b-1* and miR-27b* mimics inhibited HAdV-C5 infection. This indicated that a small subset of RNA sequences in these mimics could possibly be recognized by dsRNA sensors in a sequence specific manner. Sequence related sensing of viral RNAs have been widely studied by several groups. For example, 'GU' rich regions in the SARS-coV (SARS corona virus) ssRNA was shown to be responsible for the induction of innate immune response (Yan Li et al. 2013). The role of specific sequence motifs in the recognition of short dsRNA mimics by dsRNA sensors needs to be further studied.

A common miRNA regulatory principle is that miRNAs preferentially regulate genes that have high regulation complexity or act as nodes in topological networks (Sumazin et al. 2011). Hence by bioinformatically and experimentally elucidating the targets of the miRNAs that are down-regulated in HAdV infections, we may find elements of antiviral networks that help to restrict infection phenotypes. Such networks are of prime interest to medical sciences and important for basic biological understanding of the nature of life. Given the large complexity of the RNA world, it is also clear that many more investigations into noncoding RNA expression are needed, including miRNAs, in order to deepen our understanding of gene regulation, viral and microbial pathogenesis and innate immune response (Aderem et al. 2011). Notably, innate immunity is also at the cross road towards facilitating adaptive immunity. In addition, work from many groups have shown that miRNA regulation has a significant impact on the major cellular networks including signaling, metabolic, protein-interaction and gene regulatory networks. For example, hundreds of target mRNAs can be repressed and destabilized by a single miRNA (Krützfeldt et al. 2005; Lim et al. 2005).

We believe that this study has a significant impact and will have the answers to several key questions in the field of molecular medicine. miRNAs appear to serve as hubs of regulatory networks underlying

complex diseases (Liang 2007). Based on the experimental expression profiling data that we have obtained already, there are common genes found to be similarly regulated by different miRNAs upon transfection, such as type 1 IFN inducible genes. Clear experimental evidence for coordinated regulation of a large number of genes by miRNAs, however, is still rare. It leaves open several fundamental questions that are important for determining the value of miRNA in complex regulatory networks. This study employs a systems-based approach to understand and assemble molecular and functional regulatory networks that underlie complex physiological processes, in this case a viral infection. Complex physiological processes and infectious diseases are not determined by one gene or one pathway. Instead, networks of processes are involved, and miRNAs are an element in tuning the output of such networks. This is in part because a typical miRNA regulates dozens or hundreds of genes through binding to 3' UTR, and in part this does not require perfect complementarities between the miRNA and the target (Landgraf et al. 2007). This enables a single miRNA to regulate multiple genes in one or several pathways. In the simplest scenario, the effects are parallel, but they can also be coordinated, for example additive, synergistic, or antagonistic. Any coordinated actions on multiple target genes would provide a powerful mechanism for a single miRNA to have significant impact on a regulatory network and ultimately the physiological process or phenotype. This is exemplified in **Figure 5**. Experimental evidence for coordinated regulation of a large number of genes by miRNAs, however, is still rare. This leaves open several fundamental questions that are important for determining the value of miRNAs in complex regulatory networks. For example, if a miRNA regulates multiple genes that do not have close functional relationships, how does this miRNA achieve specificity for its effect on cellular and organ systems function? Is it possible that target genes of a particular miRNA have functional connections among them that are not yet recognized? It is possible that the specificity of the effect of a miRNA is partially determined by which target mRNAs are present in a given biological setting, which would suggest interaction between the miRNA mechanism and other mechanisms that regulate gene expression. Consistent with the notion that miRNAs are working with other mechanisms to fine- tune gene expression, many studies have shown that the effect of a miRNA on the abundance of a target is often modest. This leads to the question of the regulatory relationship between different miRNAs and between miRNAs and their protein encoding transcript and even non-coding RNA. In addition, multiple miRNAs might work together to regulate a single target, and this would result in synergistic effects.

As a major outcome, our study found a novel way to block adenovirus infection using miRNA mimics of miR-29b-1* and miR-27b*. Although, the mode of action of these miRNA mimics remains unclear

to some extent and requires further understanding, we hypothesize that these mimics exerted anti-adenoviral effect mainly through the induction of several type I IFN response genes. It is unknown if the IFN response is induced as a result of miRNA mediated direct targeting of host genes or indirectly by the activation of dsRNA sensors brought about by sensing of dsRNA mimics. However, one cannot exclude the combined effect of both these processes that gives rise to complex response.

Outlook

We aim to address several key questions in the follow up studies. We would like to document the specificity of miRNA mimics by testing several miRNA mimics ordered from different commercial vendors including the mimics with completely inactivated complementary strand to make sure that the effect of the mimics comes from a specific mature miRNA of interest. We would like to find if miR-29b-1* and miR-27b* mimics are protective for primary cells against other viruses apart from HAdV-C5 and determine which step of HAdV-C5 is specifically blocked. To address this, we perform transfection and image-based infection assays with other cell lines and other viruses. These results can be validated by determinations of viral titers produced in presence or absence of miR27b* or miR29b-1* mimics. If the results look promising, we plan to carry out infection experiments in mouse cells and in-vivo. To address which step in the infection programme of the cells are affected we plan carry out viral transcriptome analyses. These results can be compared with above mentioned bioinformatic analyses of miRNA-viral gene interactions. we would like to address if the vigorous interferon response is directly caused by targeting of genes through miRNA-mRNA targeting or by other mechanisms involving innate immunity sensors. Additionally, we would like to mutate the seed region or randomize the entire miRNA sequence without disturbing the initial nucleotide composition and test if the downregulation of HAdV-C5 targets can be attenuated, and infection can be restored. Such an approach can explain the seed specific interaction of the miRNA with its targets and also help to identify specific nucleotide motifs which can be recognized by dsRNA sensors. One can work towards developing an assay to quantitatively measure type 1 interferon, based on dual luciferase expression system under the control of an IFN sensitive and constitutive promoter, respectively. Importantly, one can also determine viral and cellular transcriptomes at different stages of infection, and compare it to non-infected, uninduced cells. If it turns out that type I IFNs are produced upon miR27b* or miR29b-1* mimics transfection, we aim to determine if the infection inhibition requires the type 1 IFN receptor in a feedback mode of action. This could be addressed using IFNR knock-out cells.

To further determine the nature of host or viral RNA targets of miR27b* or miR29b-1* mimics, one can envision to carry out RNA crosslinking and ago-pull down experiments. RNA sequences can be identified using a microarray or deep sequencing. Advanced IP-based methodology for miRNA target detection can be achieved by adding a cross-linking step to the protocol followed by high-throughput sequencing and cross-linking and immunoprecipitation (HTS–CLIP)(Chi et al. 2009), or cross-linking and immunoprecipitation coupled to high-throughput sequencing (CLIP-seq) (Zisoulis et al. 2010), or photo- activatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) (Easow et al. 2007). These methods can identify miRNA–mRNA duplexes in the immunocomplexes, as unbound RNAs are digested with nucleases, leaving those RNAs behind that are protected by the RNA silencing complex, and these will be analyzed by high- throughput RNA sequencing yielding both miRNAs and their targets.

Material and Methods

Cell Lines and Viruses

A549 human lung epithelial carcinoma cells, WI38 human embryonic lung fibroblast cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal calf serum (FCS, Invitrogen) at 37^0c under 5% CO_2 and 98% humidity. Cells were passaged every 2-4 days with a dilution of 1:2 to 1:20 and used at passage 1-25 for experiments. HAdV-C5 and HAdV-B3 were grown in A549 cells as described previously (Greber et al. 1996; Greber et al. 1993).

miRNA profiling

miRNA expression profiling of HAdV-C5 (200 MOI) and HAdV-B3 (200 MOI) infected A549 cells (300000 cells/well on a 6-well plate) was carried out in triplicates on an Agilent Microarray platform. The platform used 1899 unique miRNA sequences as probes obtained from miRBase 18. Approximately, 30 probes/miRNA were used. Total RNA was purified from the infected (24h and 48h post infection) and non-infected cells using mirVana miRNA Isolation Kit (Ambion, Austin, Texas, USA). The quality of RNA was assessed by measuring the RNA integrity number (RIN) using Bioanalyzer 2100 (Agilent, USA). Total RNA purity was determined using Nanodrop ND-1000 (Thermo Scientific, USA), (A260:A280 > 2.0; A260:A230 > 2). RNA labeling and hybridization were performed in accordance to manufacturer's indications at the Functional Genomic Centre, Zurich, Switzerland (FGCZ). Agilent scanner and the Feature Extraction 10.5 software (Agilent Technologies) were used to

obtain the microarray raw-data. Data transformation was applied to set all the negative raw values at 1.0, followed by Quantile normalization and log2 transformation. Filters on gene expression were used to keep only the miRNAs expressed in at least one sample. Agilent Human miRNA microarray results were analyzed using the GeneSpring GX 12 software (Agilent Technologies). Differentially expressed miRNAs were identified by comparing miRNA expression profiles in non-infected and HAdV infected cells. $Log_2FC > 0.5$ or $Log_2FC < -0.5$ at 24h p.i. or 48h p.i. filter and the moderated *t*-test, with $P < 0.05$ and 5% false discovery rate(FDR), were applied.

miRNA q-PCR

qRT-PCR was carried out for miR-29b-1*, miR27b*, miR-21-3p and miR-125a-5p to validate the results obtained from miRNA microarray analysis. A549 cells grown on 6-well plates (300000 cells/well) were infected with HAdV-C5 (200 MOI) and HAdV-B3 (200 MOI). 24h and 48h post infection, total RNA was extracted using mirVana RNA isolation kit (Ambion). On-column DNase digestion (Qiagen) was used to remove DNA from the samples. Total RNA purity was determined using Nanodrop ND-1000 (Thermo Scientific, USA), (A260:A280 > 2.0; A260:A230 > 2). 300 ng of total RNA was reverse transcribed using specific RT primers (50nM, **Supplementary table S7**) in 20µl reaction mixtures also containing 1× First-strand buffer (SuperScript™ III Reverse transcriptase kit, cat.no 18080044 [Invitrogen]), 0.25nM deoxynucleoside triphosphate (dNTP) mix, 10mM DTT, 4U/µl RNAseOUT (cat.no 10777019 [Invitrogen]) RNase inhibitor, and 25U/µl SuperScript III Reverse Transcriptase (cat.no 18080044 [Invitrogen]). The primers used were designed to specifically reverse transcribe the mature miRNA molecules. Reactions were incubated at 16°C for 30 min, 30°C for 30 secs, 42°C for 30 secs, 50°C for 1 sec. These steps were repeated for 60 cycles and finally incubated at 85°C for 5 min. The cDNA was amplified by PCR using Power SYBR Green Master mix (Applied Biosystems) in 10µl reaction with a pair of primers, one of which was complementary to part of the specific reverse transcription primer and the other of which was complementary to the miRNA**.** PCR reactions were carried out with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using the following cycle conditions: 95^0C for 5 minutes, followed by 40 cycles at 95^0C for 15 seconds and at 60^0C for 1 minute. Relative miRNA levels ($2^{-\Delta Ct}$) were determined by comparing the PCR quantification cycle (Ct, determined with the Software SDS 2.2.1) The expression levels of miRNAs were normalized to the geometric mean of housekeeping genes (Vandesompele et.al.200) which included 8snoRNA and U6RNA **(Primer sequences used are listed in supplementary table S7).** Dissociation plot analysis was performed to check for product specificity.

Computational analyses of miRNAs sequences

Sequence alignments between the seed region of miR-29b-1* and miR-27b* with HAdV-C5 genome were performed using a custom-made R (programming) script (will be made available upon request). Additionally, complete sequences of miR-29b-1* and miR-27b* were aligned to the 3' UTR of host factors involved in Adenovirus infection. The alignment was performed in MIRZA - online tool used for miRNA-mRNA target predictions. (Khorshid et al. 2013). Binding energy was predicted for the miRNA-mRNA target interactions by making use of the energy constants modelled on AGO PAR-CLIP data. These interactions were subsequently ranked and the scores were assigned to them.

miRNA mimics used in the transfections

All miRNA mimics used in the transfection experiments were purchased from Qiagen. Working stocks of 2µM were made for each miRNA and stored together with the 20µM stocks at -80°C. details of the mimics used in this work are listed in the **supplementary table S8**.

miRNA transfections and HAdV-C5 infection assay

Reverse transfection experiments with miR-29b-1* and miR-27b* mimics were carried out in 96-well plate format using established protocols (ThermoFisher Scientific protocols). All plates contained negative controls such as MOCK (no miRNA mimic) and siAllstar (non-targeting siRNA). Lipofectamine RNAiMAX (0.15µl, Invitrogen) was mixed with 9.85µl of OptiMEM reduced serum medium (Gibco) and added to a well containing 100nM miRNA mimic diluted in 10µl OptiMEM. The plates were incubated at room temperature (RT) for 1 h, and thereafter 8000 (A549) or 10000 (WI38) cells were added to each well in a volume of 80µl DMEM supplemented with 16% FCS, resulting in a final FCS concentration of 12.8%. Plates were incubated at 37°C for 24h. Cells were infected with HAdV-C5. The dosage of virus infection was dependent on different cell types used for infection. The infection dose used was aimed at getting about ~50% infected cells in the negative (non-transfected) control. Post infection, cells were fixed with 4% PFA (paraformaldehyde), followed by quenching with 25mM NH4Cl in 1X phosphate buffered saline (PBS). The cells were subsequently permeabilised with 0.5% Triton (TX-100) in 1X PBS for 5 min at RT. Cells were immunostained with primary rabbit anti-protein VI antibody (1:2000) (Burckhardt et al. 2011). This was followed by the incubation with specific *secondary anti-rabbit antibodies conjugated* to Alexa Fluor 488 (1:500 dilution from 2mg/ml stock) (Life Technologies) for 1hr at RT. Nuclei were stained with 4',6 - diamidino – 2 - phenylindole (DAPI) in

10% Goat Serum (GS). All the primary, and secondary antibodies used were diluted in 10% GS. After staining, plates were imaged with the Image Xpress Micro XL high throughput microscope (Molecular Devices) using 10X or 20X S Fluor objective with a 0.45 numerical aperture, 9 (3×3 grid) or 16 (4×4 grid) with no spacing and no overlap per well. DAPI stain was used to mark the cell nucleus, and a custom-made script (Matlab; Mathworks, USA) or a custom-made CellProfiler (version 2.0) pipeline was used to quantify the average nuclear intensity of the protein VI of HAdV-C5 to measure the infection index (http://cellprofiler.org) (Carpenter et al. 2006). The pipeline or the script will be made available upon request).

Gene expression profiling of miR-29b-1* and miR-27b* transfected A549 cells

10nM Qiagen miRNA mimics of miR-29b-1* miR-27b* and the non-targeting control siAllstar were reverse transfected into A549 cells. The experiment was done in 6-well plates in triplicates for the four miRNA mimics and in quadruplicates for the siAllstar control. For all, 10.9µl miRNA mimic/control siRNA stock (2µM) was diluted in 210µl OptiMEM and 221µl transfection mix composed of 3.3µl Lipofectamine RNAiMAX with 217.8µl OptiMEM was added. After 45min incubation at RT, 1749µl A549 cell suspension with 320'000cells/ml was added and the mix transferred to each well of a 6-well plate. 72h post transfection, total RNA was purified using mirVana miRNA Isolation Kit (Ambion, Austin, Texas, USA). The quality of RNA was assessed by measuring the RNA integrity number (RIN) using Bioanalyzer 2100 (Agilent, USA) and was confirmed to be above 7 in all the samples. Total RNA purity was determined using Nanodrop ND-1000 (Thermo Scientific, USA), (A260:A280 > 2.0; A260:A230 > 2). The labeling of the RNA samples for gene expression analysis was done using the protocol for the One-Color Microarray-Based Gene Expression analysis together with the Low Input Quick Amp Labeling kit (Agilent). For the labeling 1.5µl of diluted RNA samples (150ng total RNA) were used and one-color labeling was done with Cy3 (Cyanine 3 CTP, Agilent). Subsequent steps of the gene expression profiling, including hybridisation, washing and measurement was done according to the manufacturers protocol at the Functional Genomic Centre, Zurich, Switzerland (FGCZ). The chips used were custom made 4x44K arrays (Chip type: 020887, Agilent) designed in-house and contained probes for all human genes besides few probes for HAdV-B3 transcripts. The whole-genome expression data were analyzed by use of Bioconductor package in R version 3.0.1. The LIMMA package was used to identify differentially expressed genes in miR-29b-1* and mir27b* mimic transfected cells. Obtained *p*-values were adjusted for multiple testing using the false discovery rate (FDR) method; $-0.5 > Log_2FC > 0.5$ and FDR < 0.05 at 24hpi or 48hpi. Pathway enrichment analyses of

differentially expressed genes was carried out using MetaCore (Thomson Reuters) (Ekins et al. 2007).

Figure legends

Figure 1. Cellular miRNA expression profile in response to Human Adenovirus infections in human lung epithelial carcinoma (A549) cells. **A)** Heat map depicting the list of miRNAs that are strongly differentially expressed at 24h and 48h post HAdV-C5 (top) and HAdV-B3 (bottom) infection. Red denotes upregulation and blue denotes downregulation of miRNAs at particular time points. **B)** Pie Charts showing percentage of differentially regulated miRNAs for all the infection conditions. **C)** Venn diagrams showing the number of common downregulated (top panel) and upregulated (bottom panel) miRNAs between different infection conditions. **D)** qRT-PCR was performed using total RNAs extracted from the mock and HAdV-infected A549 cells. miRNA levels were normalized to 8snoRNA and U6 and expressed in fold change (n=3 per treatment). Results are expressed as mean ±SD and normalized to mock infected cells. One-way ANOVA with a Dunnett post-hoc test was used for statistical analyses; *P <0.05.

Figure 2: Predicted host and adenoviral targets of miR-29b-1*and miR-27b*. **A)** Both miR-29b-1* and miR-27b* can interact with host factors CXADR (Coxsackie virus and Adenovirus receptor) and ITGAV (alpha-V integrins) in a seed sequence-dependent or seed-independent manner. Shown are the location (position in the target) at which the miRNA could bind to the 3'UTR of the target genes, type of interaction and scores of each interaction. Strong miRNA-mRNA interactions with high binding energies are marked by high scores obtained from MIRZA scan (Khorshid et al. 2013) predictions. **B)** Viral targets were predicted by the aligning the seed sequences of miR-29b-1* (top panel) and miR-27b* (bottom panel) to the HAdV-C5 genome.

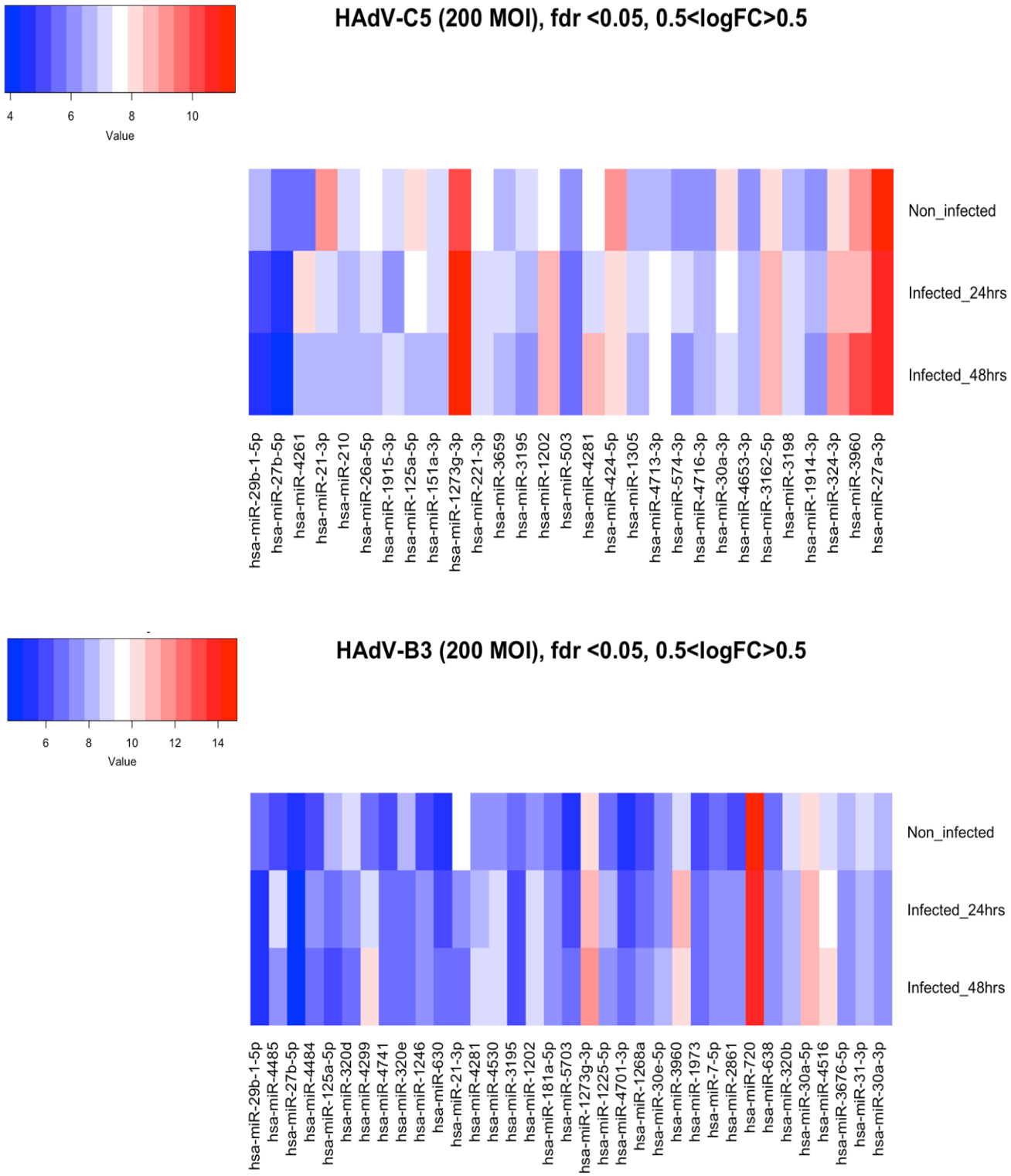
Figure 3: Effects of 10nM miRNA mimics on HAdV-C5-wt infection in A549 cells **(A)** and WI38 cells **(B)**. The cells were infected 24 h post miRNA mimic transfection and analyzed 24h post infection. Shown are infection indices (ratios of nuclei positive for the viral protein VI over total number of nuclei) and the number of nuclei analyzed. The values represent mean ± SD from three technical replicates and are compared to the controls (siAllstar transfected cells) by pairwise comparisons using Dunn's-test for multiple comparison. ***P<0.001. Statistical significance was computed only for Infection indices.

Figure 4: Transfection of A549 cells with the mimics of miR29b-1* and miR-27b* causes changes in cellular gene expression. A) Using 'limma' package for differential expression analysis, a total of 7617 genes exhibited differential expression (FDR <0.05). This represented 39% of the total 19417 transcripts on Agilent whole genome-microarray. About half of the differentially regulated genes were commonly up regulated. **B)** Venn diagram showing the overlap of differentially expressed (upregulated or down regulated) genes follwing the mimic transfection. **C)** Enrichment analysis of differentially regulated genes into gene sets. Heatmap statistics showing log2 expression values of the gene lists from the significantly enriched functional pathways (p-value $\leq$ 0.05) obtained from the pathway enrichment analysis of upregulated genes (left) and downregulated genes (right) induced by transfection of Qiagen miR29b-1* and miR-27b* mimics. Colours towards the red indicate high log_2 expression values and blue indicate low log_2 expression values of the enriched genes post transfection (shown in the colour key).

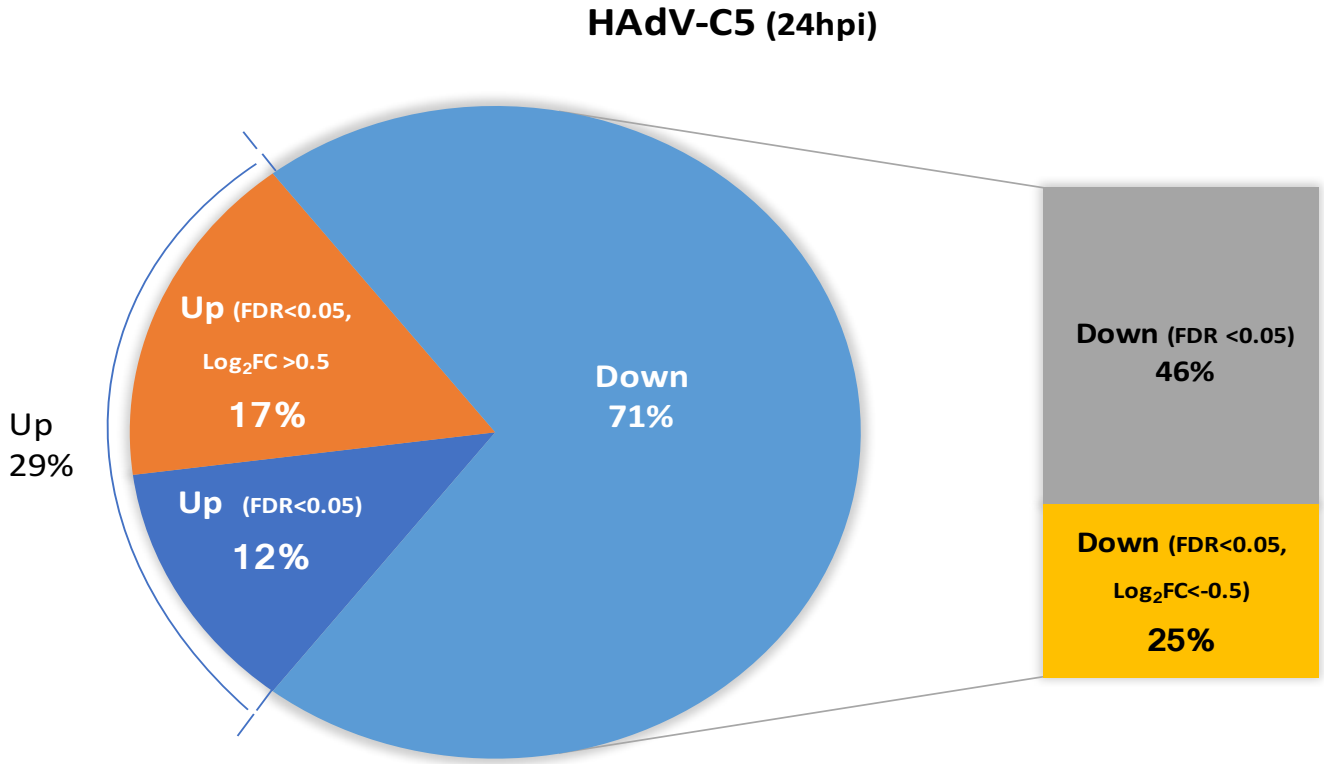
Figures

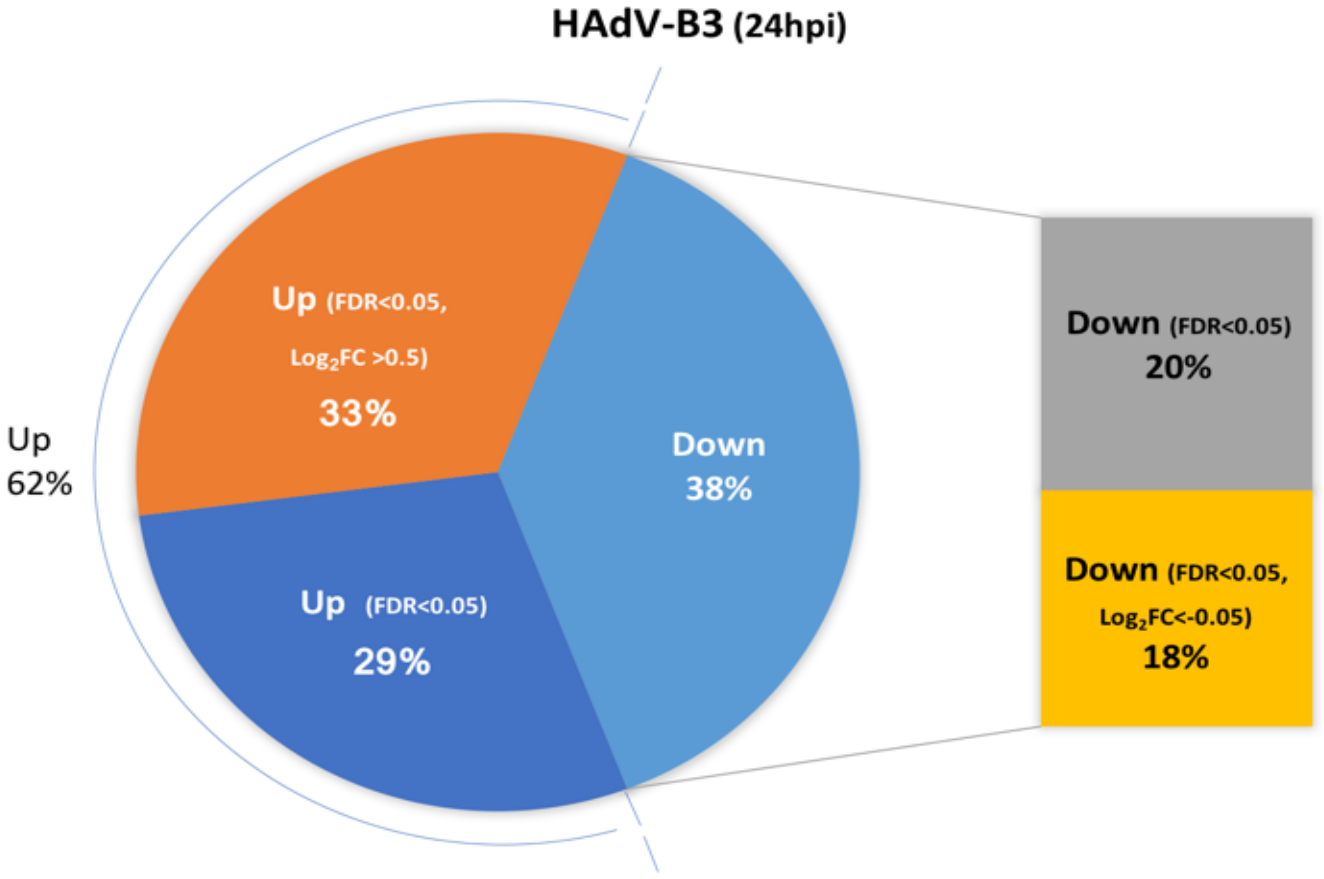
Figure 1

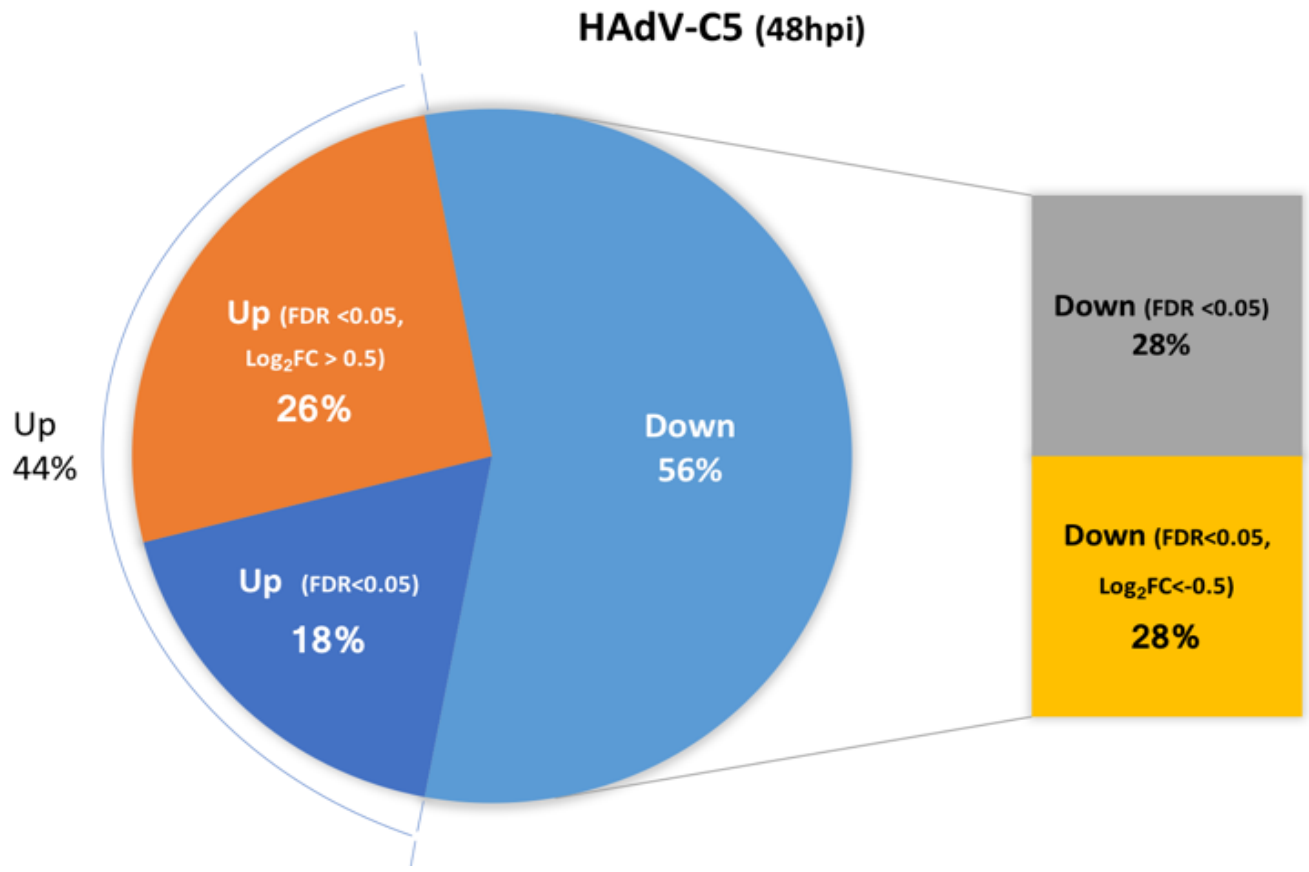
A

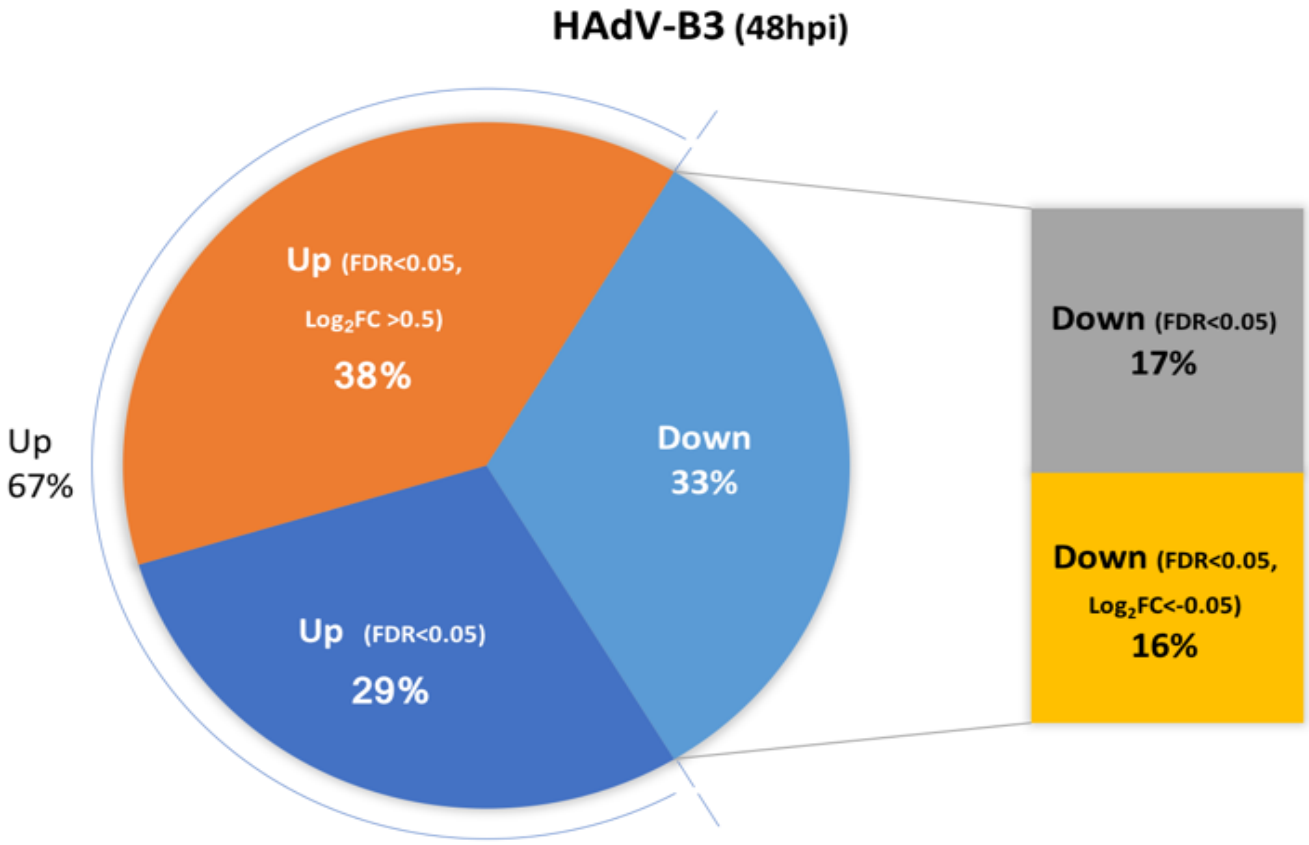


B

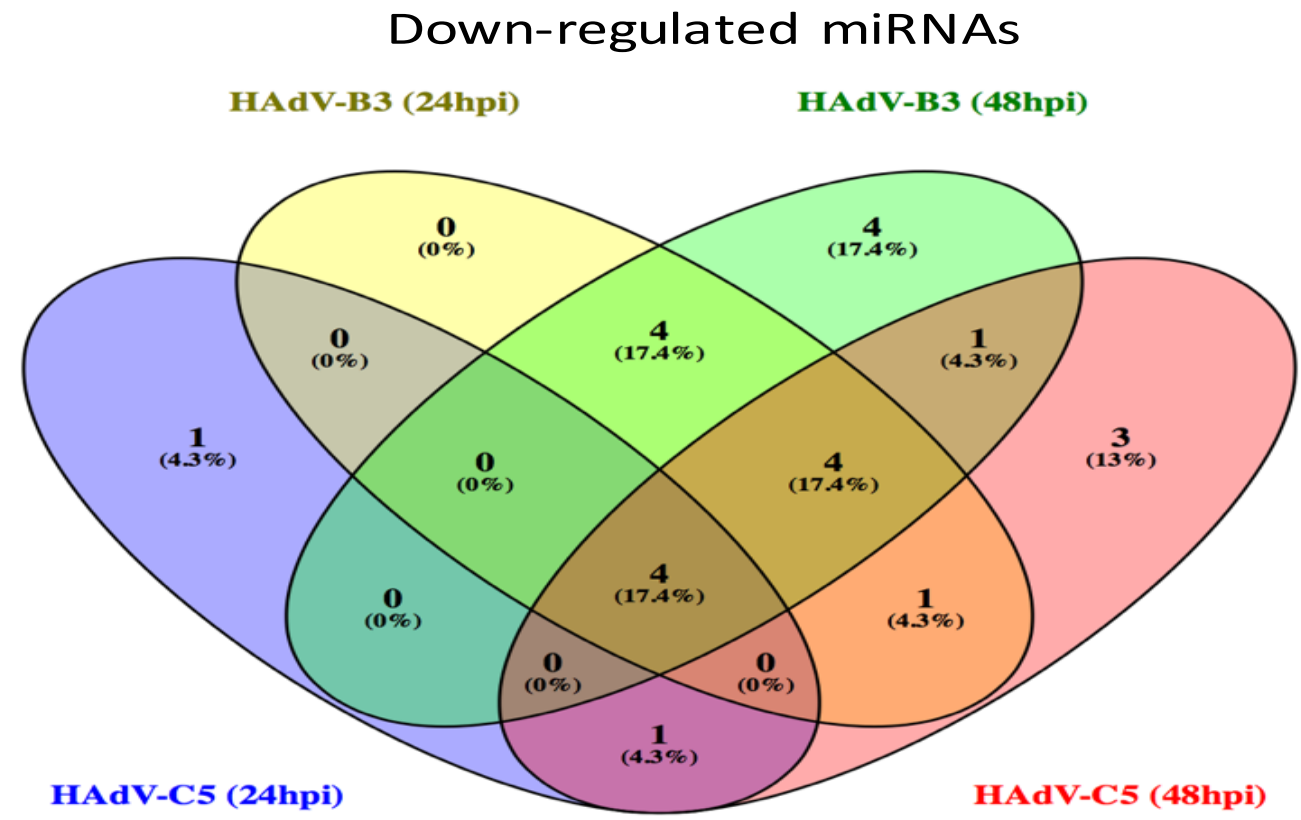

HAdV-C5 (24hpi)
Up
29%
Up (FDR<0.05, Log2FC >0.5
17%
Up (FDR<0.05)
12%
Down
71%
Down (FDR <0.05)
46%
Down (FDR<0.05, Log2FC<-0.5)
25%

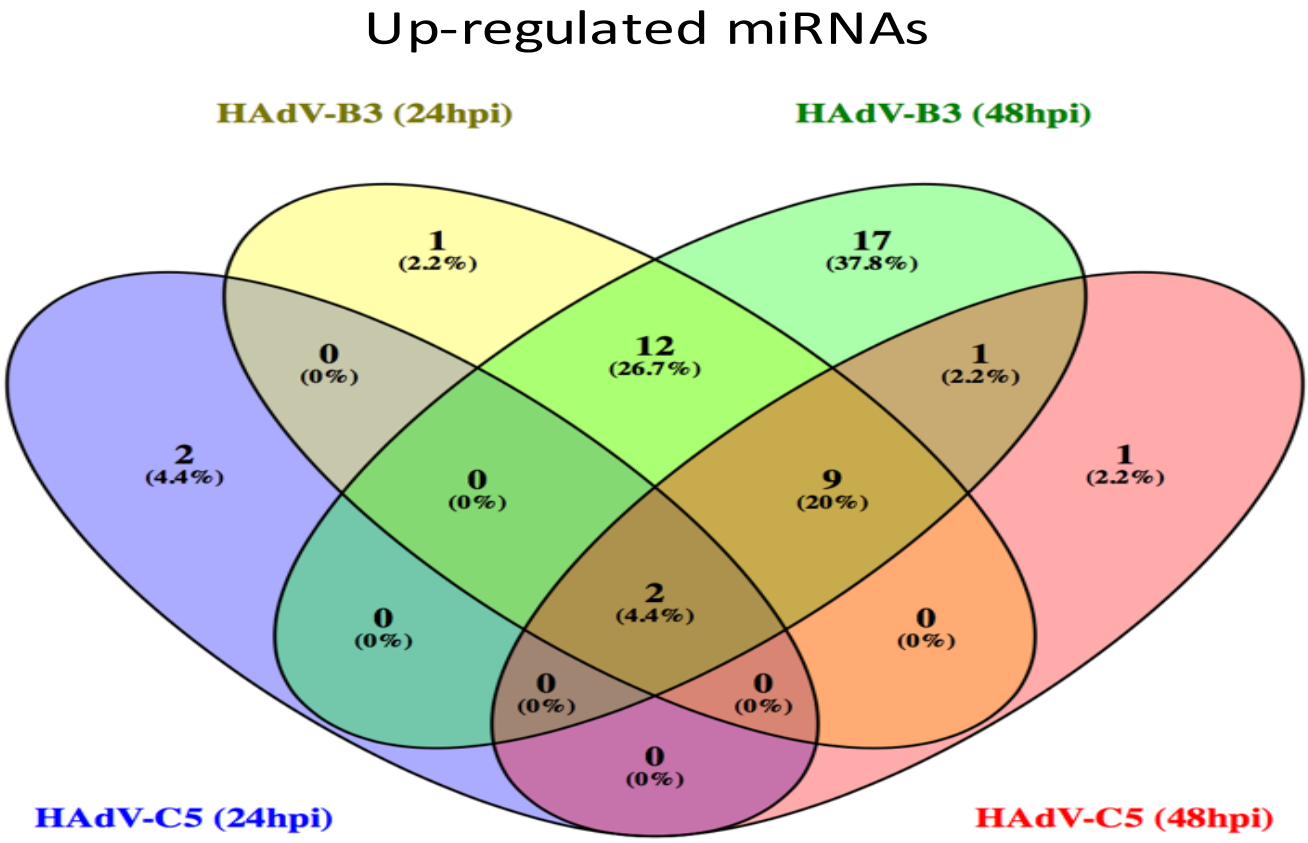

HAdV-B3 (24hpi)
Up
62%
Up (FDR<0.05, Log2FC >0.5)
33%
Up (FDR<0.05)
29%
Down
38%
Down (FDR<0.05)
20%
Down (FDR<0.05, Log2FC<-0.05)
18%


HAdV-C5 (48hpi)
Up
44%
Up (FDR <0.05, Log2FC > 0.5)
26%
Up (FDR<0.05)
18%
Down
56%
Down (FDR <0.05)
28%
Down (FDR<0.05, Log2FC<-0.5)
28%


HAdV-B3 (48hpi)
Up
67%
Up (FDR<0.05, Log2FC >0.5)
38%
Up (FDR<0.05)
29%
Down
33%
Down (FDR<0.05)
17%
Down (FDR<0.05, Log2FC<-0.05)
16%

c


Down-regulated miRNAs
HAdV-B3 (24hpi)
HAdV-B3 (48hpi)
0 (0%)
4 (17.4%)
0 (0%)
4 (17.4%)
1 (4.3%)
1 (4.3%)
0 (0%)
4 (17.4%)
3 (13%)
0 (0%)
4 (17.4%)
1 (4.3%)
0 (0%)
0 (0%)
1 (4.3%)
HAdV-C5 (24hpi)
HAdV-C5 (48hpi)


Up-regulated miRNAs
HAdV-B3 (24hpi)
HAdV-B3 (48hpi)
1 (2.2%)
17 (37.8%)
0 (0%)
12 (26.7%)
1 (2.2%)
2 (4.4%)
0 (0%)
9 (20%)
1 (2.2%)
0 (0%)
2 (4.4%)
0 (0%)
0 (0%)
0 (0%)
0 (0%)
0 (0%)
HAdV-C5 (24hpi)
HAdV-C5 (48hpi)

D

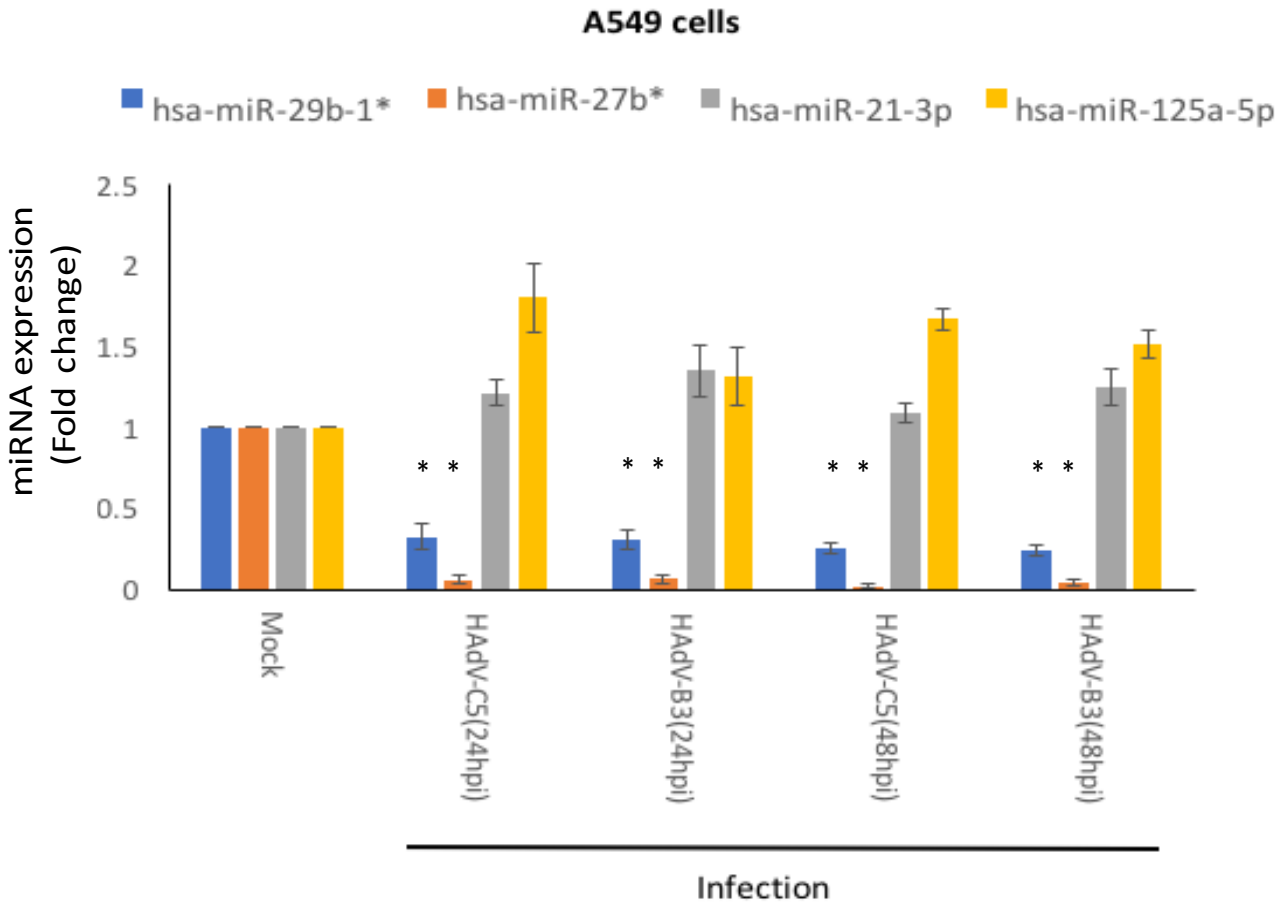

A549 cells
hsa-miR-29b-1*
hsa-miR-27b*
hsa-miR-21-3p
hsa-miR-125a-5p
miRNA expression (Fold change)
2.5
2
1.5
1
0.5
0
* *
* *
* *
* *
Mock
HAdV-C5(24hpi)
HAdV-B3(24hpi)
HAdV-C5(48hpi)
HAdV-B3(48hpi)
Infection

Figure 2

Host factor	miRNA	Location	Hybridization	Score	Interaction
CXADR	miR-29b-1*	76-125	'3 ----------UUUGGUGGUAUACUUUGGUCG---- '5 -->##########|||^^OOOOOOOOO||||||##### '5 AUUGUGUUACUAGCCUCAAAAUACAUCAAA--AAAUAAGUUAAUCAGGAACU '3	4.68937	Seed dependent
		401-450	'3 ----------UUUGGUGGU-----AUACUUUGGUCG---- '5 -->##########|||||vvvvv0000||||||##### '5 UGUGUAUUUCAUUUAUUUAUGGCCCACCAGUCUCCCCCAAAUUAGUACAG '3	1.58267	Seed dependent
		526-575	'3 ---------UUUGGUGGUAUACUUUG----GUCG---------- '5 -->#########||||^^^^00000||||vvvv|||########## '5 UAUCUCUAAAAAC----AUAGAAAACACUACAGUGGUUUAGAAAUUACUAAUUU '3	4.7598	Non-canonical
CXADR	miR-27b*	626-675	'3 ----------AAGU-GGUUA------GUCGAUUCGAGA------ '5 -->##########||vOO|||vvvvvv00000||||||###### '5 UUAGUUGAUAGACUGCUACAGGUAAUAGGGACUUAGCAAGCUCUUUUAUA '3	24.9084	Seed dependent
		1176-1225	'3 ----------AAGUGGUUA-GUCGAUUCGAGA---------- '5 -->##########||^^|||vO||||||||||########## '5 CAUGUCUCUGUGUAGUUCCAGCA--AAUCAAGCUGAGCUUUGAAAAAGUUUG '3	25.8867	Seed dependent
ITGAV	miR-29b-1*	676-725	'3 ----------UUUGGUGGUAUACUUUGGUCG-- '5 -->##########|||OOOOOOOOO|||||||### '5 AUAAAUUUUAGGUCAAAUCCUUCAAGCCAACCUAUACUAAAAAUUAGUUC '3	4.00526	Seed dependent
		1176-1225	'3 ----UUUGGUGGUAUACUUUGGUCG---------- '5 -->####|OOOOOOOOOO||||||########## '5 AAGCAAGGUAAUGUGUAAAAUCAGUCUCGGCUGUCAGAAUAACUUCUAAA '3	7.87835	Seed dependent
		2326-2375	'3 ----------UUUGGUGGUAUACUUUGGUCG---- '5 -->##########|||^^^OOOOOO|||||||##### '5 AUUUUUAUGAUCUGAUUCAGUUUUAAGAAAAC---AUGAAUGAACUAGAAGAU '3	5.53868	Seed dependent
		2726-2775	'3 ----------UUUGGUGGUAUACUUUGGUCG---------- '5 -->##########|^OOOOOOOOO|||||||########## '5 UGUGAUAAAAGUA-UUGUAUAUAAUAGAUCAGCGAUUUUUGUAAGGCAAAC '3	3.84774	Seed dependent
ITGAV	miR-27b*	126-175	'3 ----------AAGUGGUUAGUCGAUUCGAGA '5 -->##########|||OOOOOOOOO|||||||# '5 CCACAAAAUGAGAAUUAUAUUUGUCAACCUUCUCCUUAUAAAUAAGUUCA '3	10.0623	Seed dependent
		701-750	'3 ----------AAGUGGUUAGUCGAUU--CGAGA------ '5 -->##########||^O|||||^OO||vv|||||###### '5 GCCAACCUAUACUAAAAAUUAGUUCCA-UAAUCA-CAAAUGGCUCUUUUGUG '3	5.83404	Non-canonical
		1451-1500	'3 ----------AAGUGGUUA----GUCGAUU--CGAGA--------- '5 -->##########|^OOOO|||vvvvOOOO|||vv|||||######### '5 ACAUCAUGUUGUACAU-UAGAAAUGGAGAGUUUAAUAGCUCUUUAACUGCU '3	13.1153	Seed dependent
		1851-1900	'3 ----AAGUGGUUAGUCGAUUCGA---GA---------- '5 -->######||||||||||^O||||||vvv||########## '5 GCCUGCCACCAAUCA-GUAAGUUAGUCUAUAACAAAUUUUACCCUAACAGU '3	35.4559	Non-canonical

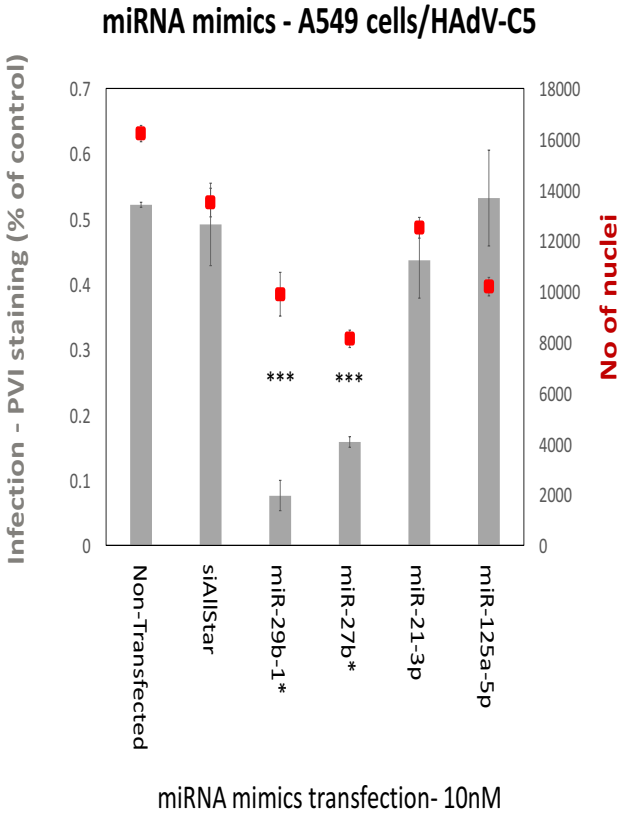
B

miR-29b-1*				
Nucleotide position from 5' end of miRNA	Sequence	Complementary HAdV sequence	Target	Region of match in mRNA
2-7	CUGGUU	GACCAA	E1B 19K	Coding region
2-8	CUGGUUU	GACCAAA	E1B 19K	Coding region
2-8	CUGGUUU	GACCAAA	pIX	3'UTR
2-8	CUGGUUU	GACCAAA	E3 12.5K	3'UTR
2-9	CUGGUUUC	CACCAAAG	pol	Coding region
2-9	CUGGUUUC	CACCAAAG	IVa2	Coding region

miR-27b*				
Nucleotide position from 5' end of miRNA	Sequence	Complementary HAdV sequence	Target	Region of match in mRNA
1.7	AGAGCUU	GACCAA	E1B 19K	Coding region
1-8	AGAGCUUA	GACCAAA	E1B 19K	Coding region
2-7	GAGCUU	GACCAAA	pIX	Coding region
2-8	GAGCUUA	GACCAAA	E3 12.5K	Coding region
2-9	GAGCUUAG	CACCAAAG	pol	Coding region

Figure 3

A



B

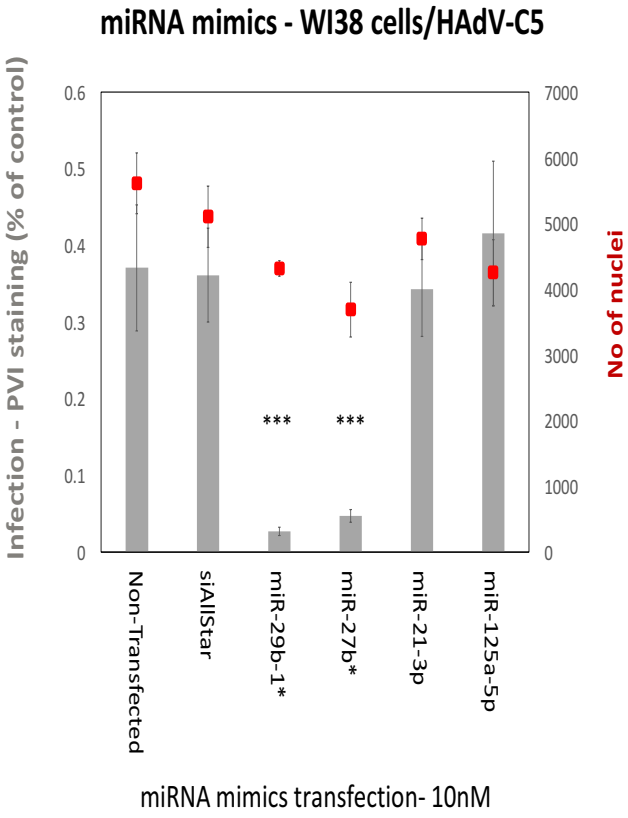
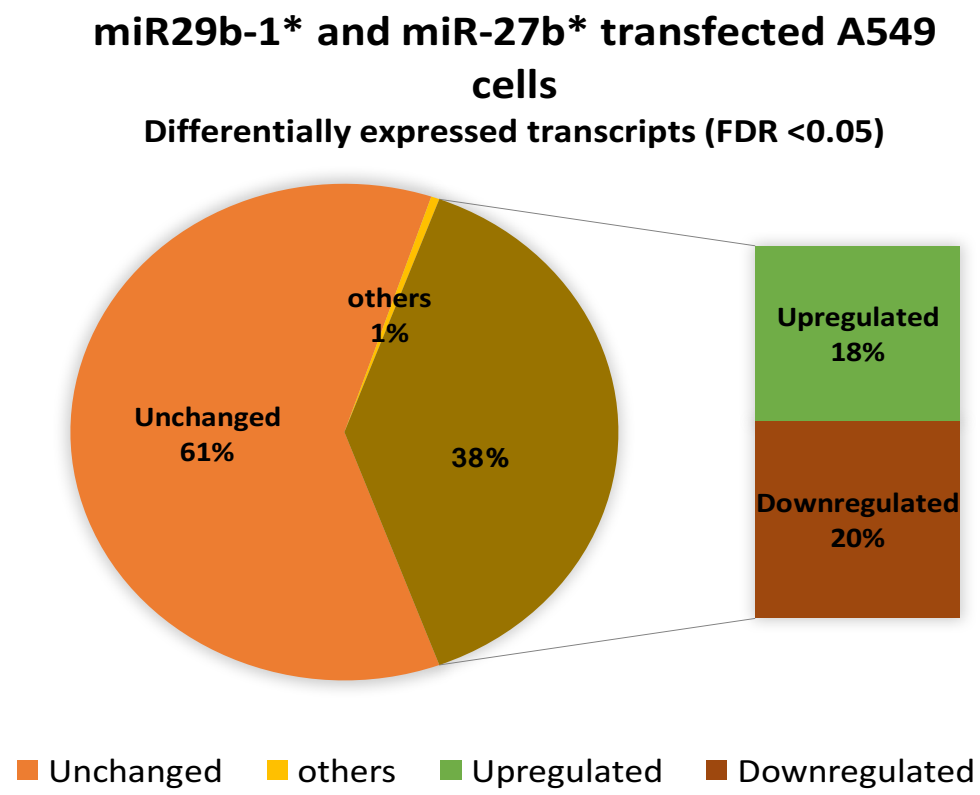
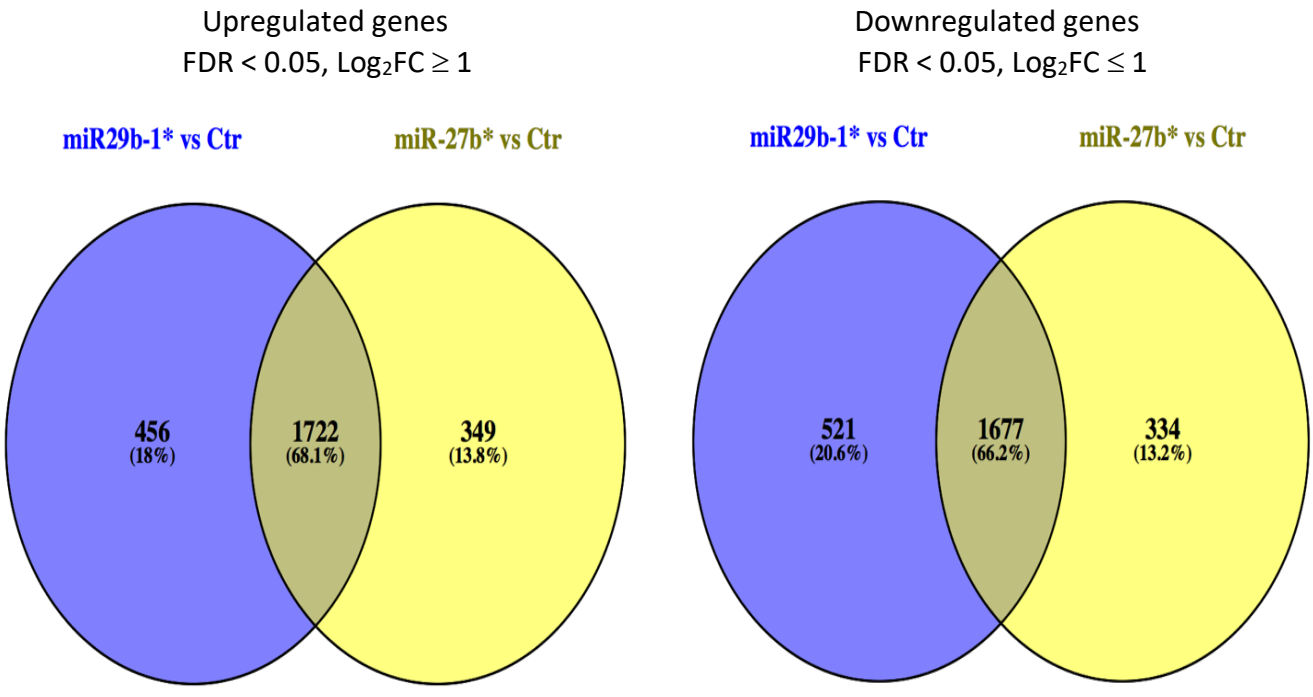


Figure 4

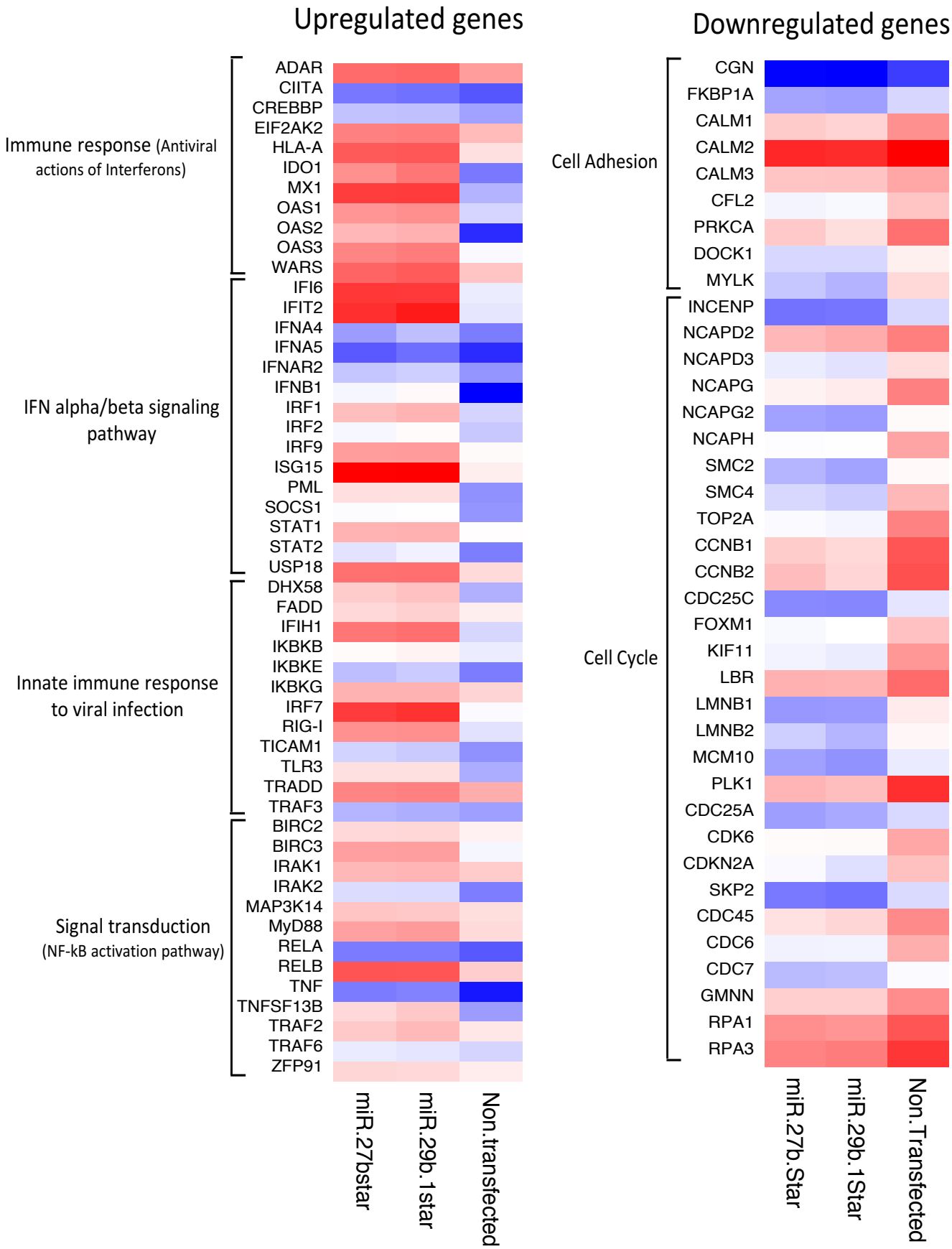
A


miR29b-1* and miR-27b* transfected A549 cells
Differentially expressed transcripts (FDR <0.05)
others 1%
Unchanged 61%
38%
Upregulated 18%
Downregulated 20%
Unchanged
others
Upregulated
Downregulated

B


Upregulated genes
FDR < 0.05, Log2FC ≥ 1
miR29b-1* vs Ctr
miR-27b* vs Ctr
456 (18%)
1722 (68.1%)
349 (13.8%)
Downregulated genes
FDR < 0.05, Log2FC ≤ 1
miR29b-1* vs Ctr
miR-27b* vs Ctr
521 (20.6%)
1677 (66.2%)
334 (13.2%)

c


Upregulated genes
Downregulated genes
Immune response (Antiviral actions of Interferons)
ADAR
CIITA
CREBBP
EIF2AK2
HLA-A
IDO1
MX1
OAS1
OAS2
OAS3
WARS
IFN alpha/beta signaling pathway
IFI6
IFIT2
IFNA4
IFNA5
IFNAR2
IFNB1
IRF1
IRF2
IRF9
ISG15
PML
SOCS1
STAT1
STAT2
USP18
Innate immune response to viral infection
DHX58
FADD
IFIH1
IKBKB
IKBKE
IKBKG
IRF7
RIG-I
TICAM1
TLR3
TRADD
TRAF3
Signal transduction (NF-kB activation pathway)
BIRC2
BIRC3
IRAK1
IRAK2
MAP3K14
MyD88
RELA
RELB
TNF
TNFSF13B
TRAF2
TRAF6
ZFP91
miR.27bstar
miR.29b.1star
Non.transfected
Cell Adhesion
CGN
FKBP1A
CALM1
CALM2
CALM3
CFL2
PRKCA
DOCK1
MYLK
Cell Cycle
INCENP
NCAPD2
NCAPD3
NCAPG
NCAPG2
NCAPH
SMC2
SMC4
TOP2A
CCNB1
CCNB2
CDC25C
FOXM1
KIF11
LBR
LMNB1
LMNB2
MCM10
PLK1
CDC25A
CDK6
CDKN2A
SKP2
CDC45
CDC6
CDC7
GMNN
RPA1
RPA3
miR.27b.Star
miR.29b.1Star
Non.Transfected

Supplementary information

Table S1: List of significantly downregulated host miRNAs upon HAdV-C5 and HAdV-B3 infection (24h and 48h p.i.) ($Log_2FC < -0.5$ at 24h or 48h p.i. and FDR < 0.05 at 24h or 48h p.i.)

HAdV-C5

Host miRNA	Log_2FC_24hr Infection	Log_2FC_48hr Infection	fdr_24hr Infection	fdr_48hr Infection
hsa-miR-21-3p	-2.282	-2.638	0.000180099	5.17E-05
hsa-miR-29b-1*	-1.482	-1.91	2.60E-06	1.28E-06
hsa-miR-125a-5p	-1.064	-1.767	0.001513903	9.19E-06
hsa-miR-27b*	-1.022	-1.686	3.15E-06	4.01E-06
hsa-miR-3676-5p	-0.481	-1.089	0.499188257	0.001398762
hsa-miR-26a-5p	-0.577	-0.931	0.000806046	6.09E-05
hsa-miR-3195	-0.347	-0.892	0.026791353	0.000125785
hsa-miR-30a-3p	-0.7	-0.825	0.162914917	0.038568781
hsa-miR-31-3p	-0.468	-0.637	0.143810681	0.028522774
hsa-miR-503	-0.414	-0.625	0.038531174	0.001789587
hsa-miR-210	-0.43	-0.608	0.000558607	0.00012939
hsa-miR-151a-3p	-0.405	-0.56	0.003332454	0.00186358
hsa-miR-374a-5p	-0.33	-0.555	0.252958965	0.009751234
hsa-miR-221-3p	-0.418	-0.513	0.014721067	0.002475247
hsa-miR-1915-3p	-0.61	0.433	0.001236674	0.004023652

HAdV-B3

Host miRNA	Log_2FC_24hr Infection	Log_2FC_48hr Infection	fdr_24hr Infection	fdr_48hr Infection
hsa-miR-21-3p	-2.102	-2.33	5.17E-05	2.95E-05
hsa-miR-125a-5p	-1.351	-2.048	7.33E-06	8.60E-07
hsa-miR-29b-1*	-1.288	-1.613	7.41E-08	7.30E-08
hsa-miR-320d	-1.171	-1.494	1.18E-05	3.50E-06
hsa-miR-320e	-1.074	-1.335	2.64E-05	2.18E-06
hsa-miR-503	-0.201	-1.293	0.003011616	0.003188094
hsa-miR-27b*	-1.137	-1.283	2.65E-07	6.83E-07
hsa-miR-720	-1.229	-1.037	0.002140303	0.003538782
hsa-miR-3195	-0.845	-1.015	0.000101008	1.14E-05
hsa-miR-30a-3p	-0.758	-0.86	0.028282381	0.012275852
hsa-miR-24-1-5p	-0.099	-0.811	0.013680255	0.008761485
hsa-miR-31-3p	-0.569	-0.712	0.023139997	0.005345675
hsa-miR-3676-5p	-0.708	-0.685	0.010526559	0.02036506
hsa-miR-320b	-0.717	-0.665	0.005707211	0.004702571
hsa-miR-18a-5p	-0.251	-0.562	0.060002749	0.005152523
hsa-miR-19b-3p	-0.488	-0.559	0.631930125	0.000210811
hsa-miR-19a-3p	-0.467	-0.548	0.751988661	0.001387747
hsa-miR-210	-0.679	-0.407	0.000293409	0.00015653

Table S2: List of significantly upregulated host miRNAs upon HAdV-C5 and HAdV-B3 infection (24h and 48h p.i.) ($Log_2FC > 0.5$ at 24h or 48h p.i. and FDR < 0.05 at 24h or 48h p.i.)

HAdV-C5

Host miRNA	Log_2FC_24hr Infection	Log_2FC_48hr Infection	fdr_24hr Infection	fdr_48hr Infection
hsa-miR-1202	0.729	0.854	0.036927531	0.008365016
hsa-miR-1273g-3p	1.365	1.502	0.004612988	0.000919009
hsa-miR-3659	0.558	0.334	0.026149739	0.784023081
hsa-miR-4261	2.535	0.71	5.76E-06	0.17405539
hsa-miR-1225-5p	0.017	0.773	0.869614395	0.002784715
hsa-miR-181a-5p	0.256	0.699	0.189323082	0.000576831
hsa-miR-197-5p	0.484	0.559	0.067232593	0.025610928
hsa-miR-2861	0.012	0.613	0.88552754	0.040111516
hsa-miR-4281	-0.38	0.849	0.043509311	0.000992851
hsa-miR-4299	0.249	1.145	0.309214329	0.000815507
hsa-miR-4516	-0.493	1.081	0.250534572	0.01570776
hsa-miR-4530	-0.043	1.222	0.753437252	0.000121166
hsa-miR-4741	0.003	1.089	0.721858581	0.000147622
hsa-miR-575	0.245	0.685	0.352741956	0.003260654
hsa-miR-638	0.048	0.755	0.859622215	0.023288543

HAdV-B3

Host miRNA	Log_2FC_24hr Infection	Log_2FC_48hr Infection	fdr_24hr Infection	fdr_48hr Infection
hsa-miR-1202	2.206	1.413	0.000109867	0.000763955
hsa-miR-1225-5p	1.991	3.215	0.000280126	0.001784177
hsa-miR-1246	1.464	1.507	3.37E-05	0.00015809
hsa-miR-1268a	1.444	1.189	0.000450941	0.004215783
hsa-miR-1273g-3p	1.433	0.995	0.000244631	0.000141758
hsa-miR-181a-5p	1.308	0.666	0.000143778	2.85E-05
hsa-miR-1973	1.242	1.66	0.000633398	0.000275348
hsa-miR-2861	1.106	1.624	0.001305924	0.000885024
hsa-miR-30a-5p	1.081	0.618	0.007639256	0.007561245
hsa-miR-30e-5p	1.081	0.899	0.000522819	5.41E-05
hsa-miR-3960	0.96	1.177	0.000616208	0.00103549
hsa-miR-4281	0.93	0.896	5.58E-05	2.05E-05
hsa-miR-4299	0.915	0.999	1.68E-05	1.20E-06
hsa-miR-4484	0.907	1.551	9.58E-06	0.000124406
hsa-miR-4485	0.828	0.791	1.26E-07	1.39E-05
hsa-miR-4516	0.771	1.124	0.00944565	0.001227899
hsa-miR-4530	0.71	1.271	0.000104835	5.99E-05
hsa-miR-4701-3p	0.706	1.001	0.000469218	0.033461578

hsa-miR-4741	0.682	1.511	2.54E-05	9.40E-06
hsa-miR-5703	0.664	0.743	0.000146906	1.62E-06
hsa-miR-630	0.62	0.96	4.74E-05	5.96E-07
hsa-miR-638	0.564	0.823	0.003344554	0.002734379
hsa-miR-7-5p	0.547	1.067	0.000652348	2.33E-06
hsa-miR-1207-5p	0.536	0.915	0.000469084	0.000666391
hsa-miR-1290	0.511	1.128	0.372871125	0.0248715
hsa-miR-197-5p	0.508	0.491	0.000469084	0.000302653
hsa-miR-222-3p	0.493	1.141	0.372871125	0.00456022
hsa-miR-23a-3p	0.491	0.64	0.050999285	0.030477345
hsa-miR-301a-3p	0.457	0.524	0.262209156	0.00440565
hsa-miR-30c-5p	0.419	1.322	0.355331417	0.006403994
hsa-miR-30d-5p	0.373	0.715	0.052663197	0.00216089
hsa-miR-3149	0.37	0.531	0.057747135	0.000175841
hsa-miR-3651	0.356	0.585	0.000314219	0.01235545
hsa-miR-4286	0.321	0.774	0.173673169	0.006004967
hsa-miR-4298	0.252	0.527	0.041766708	0.000190868
hsa-miR-4455	0.23	0.686	0.329141171	0.001207593
hsa-miR-4459	0.23	0.531	0.042769934	0.019037964
hsa-miR-4521	0.213	0.725	0.136087011	0.000224962
hsa-miR-4758-5p	0.204	0.659	0.057156457	0.000508411
hsa-miR-4800-5p	0.154	0.551	0.061318252	0.006667015
hsa-miR-483-5p	0.127	0.593	0.00874248	0.0176537
hsa-miR-574-5p	0.121	0.51	0.146121496	0.000112664

Table S3: List of top 50 significantly downregulated and upregulated host genes upon miR-29b-1* mimics transfection (72h) (FDR < 0.05)

miR-29b-1* - Downregulated host genes

GeneID	Log_2Fold change	FDR_pValue	Gene name	Gene_description
25840	-5.731	5.34E-05	METTL7A	methyltransferase like 7A
4887	-5.495	1.35E-06	NPY2R	neuropeptide Y receptor Y2
6424	-5.304	0.000221921	SFRP4	secreted frizzled-related protein 4
7276	-5.221	1.48E-05	TTR	transthyretin
643763	-5.138	5.19E-06	UG0898H09	uncharacterized LOC643763
5579	-5.125	0.031115944	PRKCB	protein kinase C, beta
23352	-4.83	7.67E-06	UBR4	ubiquitin protein ligase E3 component n-recognin 4
89919	-4.761	0.030886003	C14orf56	chromosome 14 open reading frame 56
124	-4.752	9.82E-07	ADH1A	alcohol dehydrogenase 1A (class I), alpha polypeptide
4916	-4.712	2.05E-05	NTRK3	neurotrophic tyrosine kinase, receptor, type 3
11013	-4.71	2.82E-06	TMSB15A	thymosin beta 15a
81578	-4.648	0.000242797	COL21A1	collagen, type XXI, alpha 1
126	-4.489	1.21E-05	ADH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide
79170	-4.436	0.000296926	PRR15L	proline rich 15-like
90952	-4.432	0.000258277	ESAM	endothelial cell adhesion molecule
4129	-4.411	0.000100383	MAOB	monoamine oxidase B
7429	-4.323	3.36E-06	VIL1	villin 1
11240	-4.309	0.000142596	PADI2	peptidyl arginine deiminase, type II
155006	-4.264	0.029351193	TMEM213	transmembrane protein 213
5137	-4.208	3.92E-06	PDE1C	phosphodiesterase 1C, calmodulin-dependent 70kDa
54474	-4.106	0.000224118	KRT20	keratin 20
866	-4.09	1.15E-05	SERPINA6	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6
80119	-3.985	1.84E-06	PIF1	PIF1 5'-to-3' DNA helicase homolog (S. cerevisiae)
79733	-3.976	5.69E-05	E2F8	E2F transcription factor 8
3851	-3.932	1.84E-05	KRT4	keratin 4
221357	-3.887	4.44E-05	GSTA5	glutathione S-transferase alpha 5
147381	-3.886	6.98E-05	CBLN2	cerebellin 2 precursor
56172	-3.869	1.33E-05	ANKH	ankylosis, progressive homolog (mouse)
53335	-3.853	0.041900463	BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)
114800	-3.818	0.001756483	CCDC85A	coiled-coil domain containing 85A
100169890	-3.787	0.045634698	PEG3-AS1	PEG3 antisense RNA 1 (non-protein coding)
100506627	-3.784	0.000652152	DCDC5	doublecortin domain containing 5
246213	-3.722	0.033581658	SLC17A8	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 8
10874	-3.721	5.92E-05	NMU	neuromedin U
93663	-3.679	2.33E-05	ARHGAP18	Rho GTPase activating protein 18
55789	-3.671	5.76E-05	DEPDC1B	DEP domain containing 1B
3026	-3.665	5.88E-06	HABP2	hyaluronan binding protein 2
167681	-3.639	1.61E-06	PRSS35	protease, serine, 35
90293	-3.632	0.000118498	KLHL13	kelch-like 13 (Drosophila)
57405	-3.61	2.67E-05	SPC25	SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae)
9723	-3.609	1.48E-05	SEMA3E	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E
1272	-3.605	2.70E-05	CNTN1	contactin 1
440823	-3.592	0.000371116	MIAT	myocardial infarction associated transcript (non-protein coding)
143686	-3.566	1.12E-05	SESN3	sestrin 3
345557	-3.563	3.25E-05	PLCXD3	phosphatidylinositol-specific phospholipase C, X domain containing 3
8671	-3.548	1.58E-05	SLC4A4	solute carrier family 4, sodium bicarbonate cotransporter, member 4
91057	-3.528	0.000107083	CCDC34	coiled-coil domain containing 34
1428	-3.509	1.98E-05	CRYM	crystallin, mu
3109	-3.508	1.24E-05	HLA-DMB	major histocompatibility complex, class II, DM beta
7474	-3.501	6.52E-05	WNT5A	wingless-type MMTV integration site family, member 5A

miR-29b-1* - Upregulated host genes

GeneID	Log_2Fold change	FDR_pValue	Gene name	Gene_description
6352	11.794	9.82E-07	CCL5	chemokine (C-C motif) ligand 5
282617	10.63	7.20E-08	IL28B	interleukin 28B (interferon, lambda 3)
3429	10.239	3.21E-07	IFI27	interferon, alpha-inducible protein 27
54739	10.002	1.35E-06	XAF1	XIAP associated factor 1
6288	9.905	9.01E-07	SAA1	serum amyloid A1
4939	9.556	5.51E-07	OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa
6373	9.338	1.57E-07	CXCL11	chemokine (C-X-C motif) ligand 11
684	9.2	1.57E-07	BST2	bone marrow stromal cell antigen 2
8638	9.197	1.35E-06	OASL	2'-5'-oligoadenylate synthetase-like
51296	9.055	1.33E-05	SLC15A3	solute carrier family 15, member 3
4599	8.948	9.85E-07	MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
10964	8.906	2.82E-06	IFI44L	interferon-induced protein 44-like
3620	8.901	1.00E-05	IDO1	indoleamine 2,3-dioxygenase 1
8519	8.87	9.85E-07	IFITM1	interferon induced transmembrane protein 1 (9-27)
10537	8.61	3.65E-05	UBD	ubiquitin D
3456	8.564	1.57E-07	IFNB1	interferon, beta 1, fibroblast
27074	8.552	3.15E-06	LAMP3	lysosomal-associated membrane protein 3
4600	8.507	1.11E-06	MX2	myxovirus (influenza virus) resistance 2 (mouse)
64108	8.407	2.50E-06	RTP4	receptor (chemosensory) transporter protein 4
282616	8.39	3.54E-06	IL28A	interleukin 28A (interferon, lambda 2)
3437	8.375	7.06E-07	IFIT3	interferon-induced protein with tetratricopeptide repeats 3
3627	8.347	1.57E-07	CXCL10	chemokine (C-X-C motif) ligand 10
3433	8.342	8.23E-07	IFIT2	interferon-induced protein with tetratricopeptide repeats 2
282618	8.293	2.77E-06	IL29	interleukin 29 (interferon, lambda 1)
91543	8.246	1.57E-07	RSAD2	radical S-adenosyl methionine domain containing 2
51513	8.194	5.81E-06	ETV7	ets variant 7
9636	7.796	1.71E-07	ISG15	ISG15 ubiquitin-like modifier
6866	7.715	1.03E-06	TAC3	tachykinin 3
10346	7.372	9.85E-07	TRIM22	tripartite motif containing 22
259307	7.332	9.85E-07	IL4I1	interleukin 4 induced 1
629	7.312	1.85E-07	CFB	complement factor B
5655	7.235	7.79E-07	KLK10	kallikrein-related peptidase 10
2633	7.204	4.14E-06	GBP1	guanylate binding protein 1, interferon-inducible
115362	7.109	2.61E-05	GBP5	guanylate binding protein 5
2537	7.106	3.93E-06	IFI6	interferon, alpha-inducible protein 6
3434	7.008	9.01E-07	IFIT1	interferon-induced protein with tetratricopeptide repeats 1
8843	6.963	7.46E-06	HCAR3	hydroxycarboxylic acid receptor 3
27033	6.962	1.41E-05	ZBTB32	zinc finger and BTB domain containing 32
834	6.919	1.35E-06	CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
3665	6.861	4.88E-07	IRF7	interferon regulatory factor 7
84941	6.801	2.86E-07	HSH2D	hematopoietic SH2 domain containing
1440	6.781	1.35E-06	CSF3	colony stimulating factor 3 (granulocyte)
6289	6.718	9.85E-07	SAA2	serum amyloid A2
10561	6.699	8.53E-06	IFI44	interferon-induced protein 44
116071	6.576	7.83E-07	BATF2	basic leucine zipper transcription factor, ATF-like 2
10866	6.372	1.00E-06	HCP5	HLA complex P5 (non-protein coding)
6364	6.258	0.000387248	CCL20	chemokine (C-C motif) ligand 20
7318	6.235	6.29E-06	UBA7	ubiquitin-like modifier activating enzyme 7
390035	6.191	4.00E-07	OR52K3P	olfactory receptor, family 52, subfamily K, member 3 pseudogene
6348	6.158	9.60E-05	CCL3	chemokine (C-C motif) ligand 3

Table S4: List of top 50 significantly downregulated and upregulated host genes upon miR-27b* mimics transfection (72h) (FDR < 0.05)

miR-27b* - downregulated host genes

GeneID	Log_2Fold change	FDR_pValue	Gene name	Gene_description
4887	-5.514	6.30E-05	NPY2R	neuropeptide Y receptor Y2
7276	-5.266	1.12E-06	TTR	transthyretin
56172	-4.892	0.001785565	ANKH	ankylosis, progressive homolog (mouse)
11013	-4.877	0.000149273	TMSB15A	thymosin beta 15a
81578	-4.819	0.000134199	COL21A1	collagen, type XXI, alpha 1
124	-4.783	1.23E-06	ADH1A	alcohol dehydrogenase 1A (class I), alpha polypeptide
23352	-4.78	1.09E-05	UBR4	ubiquitin protein ligase E3 component n-recognin 4
89919	-4.765	0.046362977	C14orf56	chromosome 14 open reading frame 56
11240	-4.749	1.74E-05	PADI2	peptidyl arginine deiminase, type II
3026	-4.558	0.000674034	HABP2	hyaluronan binding protein 2
4129	-4.478	0.000662567	MAOB	monoamine oxidase B
126	-4.441	1.49E-06	ADH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide
7429	-4.327	0.000202522	VIL1	villin 1
440823	-4.249	0.000168262	MIAT	myocardial infarction associated transcript (non-protein coding)
79170	-4.209	8.68E-05	PRR15L	proline rich 15-like
4916	-3.94	6.65E-05	NTRK3	neurotrophic tyrosine kinase, receptor, type 3
54474	-3.939	0.000535138	KRT20	keratin 20
80307	-3.892	1.86E-05	FER1L4	fer-1-like 4 (C. elegans) pseudogene
79733	-3.885	5.04E-05	E2F8	E2F transcription factor 8
221357	-3.849	7.02E-05	GSTA5	glutathione S-transferase alpha 5
2244	-3.843	0.000161038	FGB	fibrinogen beta chain
25840	-3.702	0.00046779	METTL7A	methyltransferase like 7A
80119	-3.628	6.67E-06	PIF1	PIF1 5'-to-3' DNA helicase homolog (S. cerevisiae)
57405	-3.626	1.71E-05	SPC25	SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae)
2939	-3.621	2.98E-05	GSTA2	glutathione S-transferase alpha 2
29993	-3.595	1.74E-05	PACSIN1	protein kinase C and casein kinase substrate in neurons 1
114800	-3.59	0.00198809	CCDC85A	coiled-coil domain containing 85A
167681	-3.588	0.000202522	PRSS35	protease, serine, 35
6424	-3.587	1.18E-05	SFRP4	secreted frizzled-related protein 4
5137	-3.578	4.90E-05	PDE1C	phosphodiesterase 1C, calmodulin-dependent 70kDa
10874	-3.559	2.09E-05	NMU	neuromedin U
93663	-3.559	0.000119066	ARHGAP18	Rho GTPase activating protein 18
48	-3.526	3.73E-06	ACO1	aconitase 1, soluble
79923	-3.513	0.041186968	NANOG	Nanog homeobox
100505683	-3.501	5.07E-05	LOC100505683	uncharacterized LOC100505683
866	-3.451	7.33E-05	SERPINA6	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6
83896	-3.419	0.000180165	KRTAP3-1	keratin associated protein 3-1
26190	-3.391	0.001323991	FBXW2	F-box and WD repeat domain containing 2
55304	-3.372	0.006940347	SPTLC3	serine palmitoyltransferase, long chain base subunit 3
9452	-3.365	2.85E-05	ITM2A	integral membrane protein 2A
1361	-3.346	1.88E-05	CPB2	carboxypeptidase B2 (plasma)
486	-3.343	1.44E-05	FXYD2	FXYD domain containing ion transport regulator 2
3109	-3.339	1.44E-05	HLA-DMB	major histocompatibility complex, class II, DM beta
119587	-3.321	0.026234395	CPXM2	carboxypeptidase X (M14 family), member 2
3488	-3.319	0.001459134	IGFBP5	insulin-like growth factor binding protein 5
7474	-3.304	9.71E-05	WNT5A	wingless-type MMTV integration site family, member 5A
5239	-3.295	1.83E-05	PGM5	phosphoglucomutase 5
7095	-3.283	0.000236942	SEC62	SEC62 homolog (S. cerevisiae)
147381	-3.274	0.000581525	CBLN2	cerebellin 2 precursor
151258	-3.242	0.000614474	SLC38A11	solute carrier family 38, member 11

miR-27b* - upregulated host genes

GeneID	Log_2Fold change	FDR_pValue	Gene name	Gene_description
6352	11.772	1.55E-06	CCL5	chemokine (C-C motif) ligand 5
54739	10.915	6.79E-07	XAF1	XIAP associated factor 1
3429	10.357	2.44E-07	IFI27	interferon, alpha-inducible protein 27
282617	10.062	2.44E-07	IL28B	interleukin 28B (interferon, lambda 3)
4939	9.307	1.12E-06	OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa
8638	9.123	2.74E-06	OASL	2'-5'-oligoadenylate synthetase-like
51296	8.941	1.86E-05	SLC15A3	solute carrier family 15, member 3
4599	8.858	1.51E-06	MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
10964	8.705	7.60E-06	IFI44L	interferon-induced protein 44-like
684	8.636	2.44E-07	BST2	bone marrow stromal cell antigen 2
6373	8.546	2.44E-07	CXCL11	chemokine (C-X-C motif) ligand 11
8519	8.531	1.51E-06	IFITM1	interferon induced transmembrane protein 1 (9-27)
6288	8.506	2.06E-06	SAA1	serum amyloid A1
10537	8.284	5.83E-05	UBD	ubiquitin D
27074	8.274	6.23E-06	LAMP3	lysosomal-associated membrane protein 3
4600	8.255	2.00E-06	MX2	myxovirus (influenza virus) resistance 2 (mouse)
91543	8.12	2.44E-07	RSAD2	radical S-adenosyl methionine domain containing 2
64108	8.08	6.82E-06	RTP4	receptor (chemosensory) transporter protein 4
3456	8.058	2.44E-07	IFNB1	interferon, beta 1, fibroblast
3620	8.052	2.41E-05	IDO1	indoleamine 2,3-dioxygenase 1
3437	7.836	1.57E-06	IFIT3	interferon-induced protein with tetratricopeptide repeats 3
282616	7.828	8.00E-06	IL28A	interleukin 28A (interferon, lambda 2)
282618	7.827	6.25E-06	IL29	interleukin 29 (interferon, lambda 1)
51513	7.781	8.76E-06	ETV7	ets variant 7
9636	7.749	2.48E-07	ISG15	ISG15 ubiquitin-like modifier
3433	7.645	7.79E-07	IFIT2	interferon-induced protein with tetratricopeptide repeats 2
2633	7.578	7.60E-06	GBP1	guanylate binding protein 1, interferon-inducible
3627	7.385	5.35E-07	CXCL10	chemokine (C-X-C motif) ligand 10
2537	7.199	6.26E-06	IFI6	interferon, alpha-inducible protein 6
10346	7.141	1.30E-06	TRIM22	tripartite motif containing 22
6866	7.02	1.51E-06	TAC3	tachykinin 3
5655	6.945	1.05E-06	KLK10	kallikrein-related peptidase 10
8843	6.937	1.33E-05	HCAR3	hydroxycarboxylic acid receptor 3
115362	6.873	4.86E-05	GBP5	guanylate binding protein 5
3434	6.708	1.34E-06	IFIT1	interferon-induced protein with tetratricopeptide repeats 1
834	6.608	3.99E-06	CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
3665	6.581	9.25E-07	IRF7	interferon regulatory factor 7
27033	6.573	2.76E-05	ZBTB32	zinc finger and BTB domain containing 32
629	6.534	5.35E-07	CFB	complement factor B
10561	6.483	1.54E-05	IFI44	interferon-induced protein 44
259307	6.441	2.06E-06	IL4I1	interleukin 4 induced 1
84941	6.398	1.30E-06	HSH2D	hematopoietic SH2 domain containing
6364	6.265	0.000594301	CCL20	chemokine (C-C motif) ligand 20
116071	6.248	1.05E-06	BATF2	basic leucine zipper transcription factor, ATF-like 2
10866	6.13	1.52E-06	HCP5	HLA complex P5 (non-protein coding)
10202	6.05	1.39E-06	DHRS2	dehydrogenase/reductase (SDR family) member 2
352999	6.041	1.60E-05	C6orf58	chromosome 6 open reading frame 58
7318	5.954	1.31E-05	UBA7	ubiquitin-like modifier activating enzyme 7
3965	5.857	8.91E-06	LGALS9	lectin, galactoside-binding, soluble, 9
64135	5.856	1.31E-05	IFIH1	interferon induced with helicase C domain 1

Table S5: list of significant-scoring pathway maps (p.value < 0-05**) of upregulated genes upon miR-29b-1* and miR-27b* mimic (72h) transfection obtained from MetaCore enrichment analysis.**

miR-29b-1*

Maps	Total no of genes in the pathway	pValue	No of differentially upregulated genes from this pathway	list of differentially upregulated genes from the pathway
Immune response (*IFN alpha/beta signaling pathway*)	24	9.69E-06	10	PML, ISG15, IFI6, IFN-alpha, SOCS1, IRF2, IRF9, IRF1, IFN-beta, USP18
Immune response (*Role of PKR in stress-induced antiviral cell response*)	57	1.71E-04	14	p38alpha (MAPK14), TRAF3, ATF-2, TRAF2, IFN-alpha, IL-8, TRAF6, IRF7, IRF3, TRIF (TICAM1), IRF1, TLR3, IFN-beta, TNF-alpha
Innate immune response to viral infection	28	2.88E-04	9	TRAF3, IFN-alpha, IL-8, LGP2, IRF7, IRF3, TRADD, TLR3, IFN-beta
Immune response (*Classical complement pathway*)	53	1.08E-03	12	C5aR, C1r, C3, C3b, C3c, C2, iC3b, C3dg, C2b, C3a, C1s, C2a
Immune response (*IFN gamma signaling pathway*)	56	5.56E-03	11	CBP, VCAM1, ATF-2, SMAD7, SOCS1, IRF9, CSF1, IRF1, p300, p38 MAPK, AFAP
Immune response (*Antiviral actions of Interferons*)	52	9.35E-03	10	CBP, INDO, WARS, IFN-alpha, ADAR1, IRF9, IRF3, IRF1, p300, IFN-beta
Signal transduction (*NF-kB activation pathways*)	51	2.28E-02	9	Ubiquitin, c-IAP2, TRAF3, TRAF2, c-IAP1, TRAF6, TRADD, ZFP91, TNF-alpha

miR-27b*

Maps	Total no of genes in the pathway	pValue	No of differentially upregulated genes from this pathway	list of differentially upregulated genes from the pathway
Immune response_IFN alpha/beta signaling pathway	24	1.44E-11	17	STAT2, PML, ISG15, IFI6, IFN-alpha, SHP-2,ISGF3, IFNAR2,STAT1/STAT2, SOCS1, IRF2, IRF9, PTP-1B, IRF1, IFN-beta, STAT1, USP18
Immune response_Role of PKR in stress-induced antiviral cell response	57	6.18E-10	25	I-kB, Caspase-7, TRAF3, MyD88, PKR, RelA (p65 NF-kB subunit),TLR3 ATF-2, TRAF2, IFN-alpha, TRAF6, IKK-beta, c-Jun, IRF7, NFKBIB, IFN-gamma receptor, NF-kB, TRIF (TICAM1), IRF1, Caspase-8, IFN-beta, NFKBIA, STAT1, ATF-2/c-Jun, TNF-alpha
Immune response_Innate immune response to RNA viral infection	28	6.40E-09	16	IKK-epsilon, I-kB, Caspase-10, TRAF3, MDA-5, IFN-alpha, IKK-beta, LGP2, IRF7, TRADD, NF-kB, FADD, RIG-I, Caspase-8, TLR3, IFN-beta
Signal transduction_NF-kB activation pathways	51	1.53E-06	19	Ubiquitin, I-kB, NF-kB2 (p100), RelA (p65 NF-kB subunit), TRAF3, MyD88, NF-kB2 (p52), TRAF2, TRAF6, IKK-beta, NF-kB p52/RelB, TRADD, NF-kB, NIK(MAP3K14), RelB(NF-kB subunit), ZFP91, IRAK1, TNF-alpha, IRAK2,
Immune response _IFN gamma signaling pathway	56	1.76E-06	20	CBP, VCAM1, PKR, IP3 receptor MEKK1(MAP3K1), ATF-2, AFAP, C/EBPbeta, SMAD7, SHP-2, ISGF3, SOCS1, IRF9, IFN-gamma receptor, CSF1, IRF1, C3G, PDK (PDPK1), p38 MAPK, STAT1,
Immune response_Antiviral actions of Interferons	52	9.60E-06	18	OAS1, CBP, INDO, MxA, STAT2, MHC class II, WARS, PKR, IFN-alpha, ISGF3, CIITA, OAS3, IRF9, IFN-beta, IFN-gamma receptor, IRF1, STAT1 2'-5'-oligoadenylate synthetase
Immune response_Classical complement pathway	53	2.03E-03	14	C5aR, C4BP, C1 inhibitor, C3, C3b, C3c, C2, DAF, C8gamma, iC3b, C2b, C3a, C2a, C3dg,

Table S6: list of significant-scoring pathway maps (p.value < 0-05**) of downregulated genes upon miR-29b-1* and miR-27b* mimic (72h) transfection obtained from MetaCore enrichment analysis.**

miR-29b-1*

Maps	Total no of genes in the pathway	pValue	No of differentially upregulated genes from this pathway	list of differentially upregulated genes from the pathway
Cell cycle (*Chromosome condensation in prometaphase*)	21	3.97E-10	16	Cyclin B, Histone H3, CAP-G, E140CAP-G/G2, INCENP, CAP-C, CAP-D2/D3, Aurora-A, CAP-E, Condensin, CAP-H/H2, CNAP1, Aurora-B, TOP2, BRRN1, Histone H1
Cell cycle (*Start of DNA replication in early S phase*)	32	5.12E-08	18	MCM3, CDC7, ORC1L, MCM5, RPA3, MCM4, MCM10, ASK (Dbf4), CDC45L Geminin, CDC18L (CDC6), Cyclin E, RPA1, ORC5L, MCM4/6/7 complex, MCM2, HP1 alpha, Histone H1
Cell cycle (*Initiation of mitosis*)	26	4.36E-07	15	Cyclin B2, Histone H3, CDC25C, FOXM1, Cyclin H, Nucleolin, Wee1, Kinase MYT1, Lamin B, KNSL1,MAT1, AKT(PKB),Cyclin B1, PLK1,Histone H1
Cell adhesion (*Histamine H1 receptor signaling in the interruption of cell barrier integrity*)	45	6.56E-06	19	MLCP (cat), ZO-1, Beta-catenin, CPI-17, Alpha-catenin, Paxillin, IP3 receptor, ROCK, Calmodulin, PLC-beta, Vinculin, PKC-alpha, MLCK, Occludin, G-protein beta/gamma, Actin cytoskeletal, MELC, Cofilin p120-catenin
Cell adhesion (*Gap junctions*)	30	2.35E-03	11	Tubulin beta, Cingulin, Connexin 32, ZO-1, Connexin 50, Actin, Caveolin-1 Tubulin alpha, PKC, Occludin, Tubulin (in microtubules)
Cell cycle (*Regulation of G1/S transition*)	38	1.71E-02	11	TGF-beta 2, PP2A regulatory, CDC25A, SMAD2, CDK6, GSK3 beta, p70 S6 kinase1, Cyclin E, p16INK4, Skp2, TGF-beta receptor type I
Cell adhesion (*Integrin-mediated cell adhesion and migration*)	48	1.76E-02	13	MLCP (cat), DOCK1, MyHC, Paxillin, ROCK, alpha-10/beta-1 integrin, Vinculin, Actin cytoskeletal, Cofilin Collagen IV, MELC, MYLK1, MLCK

miR-27b*

Maps	Total no of genes in the pathway	pValue	No of differentially upregulated genes from this pathway	list of differentially upregulated genes from the pathway
Cell cycle (*Chromosome condensation in prometaphase*)	21	6.51E-12	17	Cyclin B, Histone H3, CAP-G, CNAP1, CAP-G/G2, INCENP, CAP-C, CAP-E, CAP-D2/D3, Aurora-A, AKAP8, Condensin, CAP-H/H2, BRRN1, Aurora-B, TOP2, Histone H1
Cell cycle (*Start of DNA replication in early S phase*)	32	1.38E-07	17	MCM3, CDC7, ORC1L, MCM5, RPA3, MCM4, MCM10, Geminin, CDC18L (CDC6), Cyclin E, RPA1, ORC5L, MCM4/6/7 complex, MCM2, HP1 alpha, Histone H1, CDC45L
Cell cycle (*Initiation of mitosis*)	26	1.45E-06	14	Cyclin B2, Histone H3, CDC25C, FOXM1, Cyclin H, Nucleolin, Wee1, Kinase MYT1, Lamin B, KNSL1, Cyclin B1, PLK1, MAT1, Histone H1
Cell adhesion (*Histamine H1 receptor signaling in the interruption of cell barrier integrity*)	45	6.23E-04	15	MLCP (cat), Alpha-actinin, CPI-17, Alpha-catenin, Paxillin, IP3 receptor, Calmodulin, PLC-beta, PKC-alpha, Occludin, G-protein beta/gamma, MELC, p120-catenin, MLCK, Cofilin
Cell adhesion (*Gap junctions*)	30	1.06E-02	7	Tubulin beta, Cingulin, Connexin 32, Tubulin alpha, PKC, Occludin, Tubulin (in microtubules)
Cell cycle (*Regulation of G1/S transition*)	38	1.36E-02	9	PP2A regulatory, CDC25A, SMAD2, CDK6, GSK3 beta, p70 S6 kinase1, Cyclin E, Skp2/TrCP/FBXW, p16INK4

Table S7: Primers used in qRT- PCR validations of miRNA microarray results

Primer Name	Primer Sequences 5'-> 3'	Type
hsa-miR-27b*_RT	GCA TAC TCC GAC CGT TAC TGT TCA C	Reverse Transcriptase Primer
hsa-miR-27b*_Forward	AGG CAT ACT CCG ACC GTT ACT G	Forward primer
hsa-miR-27b*_Reverse	ACG GTG GAG AGC TTA GCT GAT TG	Reverse primer
hsa-miR-29b-1*_RT	GCA TAC TCC GAC CGT TAC TTC TAA A	Reverse Transcriptase Primer
hsa-miR-29b-1*_Forward	TCA GCA TAC TCC GAC CGT TAC T	Forward primer
hsa-miR-29b-1*_Reverse	GGT GCG CTG GTT TCA TAT GGT	Reverse primer
hsa-miR-21-3p_RT	TCC TCA GGT CGA ACC TAT TGA CAG C	Reverse Transcriptase Primer
hsa-miR-21-3p_Forward	GCT CCT CAG GT GAA CCT ATT G	Forward primer
hsa-miR-21-3p_Reverse	CCG ACG CCA ACA CCA GTC GAT	Reverse primer
hsa-miR-125a-5p_RT	CCT CAC AAC GAT TCC ACA AGC ACA G	Reverse Transcriptase Primer
hsa-miR-125a-5p_Forward	GTC CTC ACA ACG ATT CCA CAA G	Forward primer
hsa-miR-125a-5p_Reverse	GTT GAT TCT CCC TGA GAC CCT TTA	Reverse primer
U6RNA_Forward	CTCGCTTCGGCAGCACA	Forward primer
U6RNA_reverse	AACGCTTCACGAATTTGCGT	Reverse primer
sno8RNA_forward	GCATGGGTTTGGATTTATGATGG	Forward primer
sno8RNA_reverse	GGTTCAGGGCTACAGTTTGC	Reverse primer

Table S8: list of miRNA mimics and siRNA used in the study

miRNA mimic	Sequence 5' -> 3' / comments	ID / Cat. Nr.
Syn-hsa-miR-27b* miScript miRNA Mimic	AGAGCUUAGCUGAUUGGUGAAC	MSY0004588
Syn-hsa-miR-29b-1* miScript miRNA Mimic	GCUGGUUUCAUAUGGUGGUUUAGA	MSY0004514
Syn-hsa-miR-21-3p miScript miRNA Mimic	CAACACCAGUCGAUGGGCUGU	MSY0004494
Syn-hsa-miR-125a-5p miScript miRNA Mimic	UCCCUGAGACCCUUUAACCUGUGA	MSY0000443
AllStars Negative Control siRNA (5 nmol)	nonsilencing siRNA	1027280

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Chapter 3

Third chapter gives details about the 2nd project carried out during my PhD. The main aim of the project was to address several key questions raised from previous projects as discussed in the outlook section of **chapter 2**, and to carry out follow up studies. The results obtained from this project clearly suggest that the mimics of miR-29b-1* and miR-27b* did not function as bonafide miRNAs. Yet, mimics of these miRNAs inhibited several viral infections. These mimics were found to promote antiviral sensing in cultured cells through the activation of dsRNA sensor - RIG-I. This project mainly discusses the mode of action of these miRNA mimics in activating RIG-I. The experiments included transient transfections, infection assays, IFN measurements, siRNA mediated knockdown assays and NF-kB translocation assay. This chapter has following sections: 1) Abstract, 2) Introduction to the topic, 3) Results derived from the experiments, 4) Discussion of the results, outlook and importance of this work, and 5) Experimental procedures used in this project.

Chapter 3

Cellular miRNA-like molecules promote antiviral sensing in cultured cells

(Manuscript in preparation)

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Abstract

Viruses are a worldwide health threat, and for many viruses antiviral treatments or vaccines are insufficient. Mammalian host cells have defence mechanisms to suppress virus infections. Non-self, double stranded RNAs (dsRNA) are well known inducers of antiviral innate Immune responses. dsRNA-mediated host cell responses and subsequent Interferon induction serve as an important defense mechanism against virus infections. In this study, we describe an antiviral effect of sequence-specific, chemically synthesized non-modified, blunt-ended, double-stranded microRNA-like molecules known as miRNA mimics. Two blunt-ended mimics with miR-29b-1* and miR-27b* nucleotide sequence significantly suppressed several infections with human adenovirus 5 (HAdV-C5), Influenza A virus (IAV- H3N2[X31 strain]), human rhinovirus (HRV1A), Semliki forest virus (SFV) and vesicular stomatitis virus (VSV)) of cell cultures, but the same sequences with 3' overhangs did not have antiviral effects. This suggests that the blunt-end miR-29b-1* and miR-27b* mimics did not function as true host miRNAs to elicit the anti-virus response. Further experiments indicated that the mimics directly or indirectly activated the dsRNA sensor Retinoic acid-inducible gene I (RIG-I), induced Interferon α/β secretion via MAVS and IRF3, and thereby gave rise to antiviral immune response. These findings suggest that, blunt-ended, non-modified dsRNA molecules bearing sequence similarity to miR-29b-1* and miR-27b can potentially be developed into broad antiviral drugs.

Introduction

Viruses are major etiological agents of disease in humans. They have diverse structures, replication strategies and elicit complex interactions with the host cell. These features have been exploited for the development of antiviral drugs and vaccines. Currently available methods which aim at inhibiting the infection and minimizing the viral spread depend on several factors such as availability of vaccines, drug susceptibility, mode of transmission, epidemiology etc. Viruses have fast mutation rates (particularly in case of RNA viruses) and hence the currently available antiviral medications targeting the viral proteins have limited shelf life and narrow scope of attack. The prospects of using siRNA-based (Wittrup & Lieberman 2015) and miRNA-based therapeutics (Broderick & Zamore 2011) against viral infection is has been gaining a lot of importance over the last few years. The amount of specific dsRNA in the host cells can be increased considerably through the transfection of synthetic molecules that trigger RNAi (Denli & Hannon 2003; Kim 2003; Elbashir et al. 2001). Most of the times, short dsRNA fragments (22-24nt) are generated upon cleavage of long dsRNA by DICER (endoribonuclease with RNase motif). To bypass the above step, chemically synthesized short dsRNA can also be introduced into the cells directly via electroporation or transfection. dsRNA has been used with a lot of success against several DNA and RNA viruses (Barik 2004; Wittrup & Lieberman 2015). They can block the viral replication, inhibit the infection by activating the host innate immune response or by directly targeting the essential host or viral genes that are involved in virus life cycle.

MicroRNA (miRNA) mimics (approx. 22 – 24nucleotides) represent a form of short non-self, chemically synthesized double stranded RNA (dsRNA). They act as functional equivalents to endogenous human miRNAs. Previous studies have shown that non-self dsRNA molecules can be recognized and sensed by host innate immune system through specific cytosolic pattern recognition receptors (PRRs) (Uematsu & Akira 2008; Karpala et al. 2005). PRRs for dsRNA include toll-like receptors (TLR3) (localized on the cell surface or in endosomes) (Helen Flo & Aderem 2005) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) (Yoneyama et al. 2004) or melanoma differentiation-associated gene 5 (MDA5) (Yoneyama et al. 2005). Caspase recruitment domains (CARDs) and DExD/H-box helicase domains form the main constituents of RIG-I and MDA5 sensing. Mitochondrial antiviral signalling protein (MAVS) is a downstream effector of MDA5 and RIG-I activation. Upon sensing of dsRNA, RIG-I or MDA5 CARD domain interacts with the CARD domains of MAVS (Hiscott et al. 2006) and this mediates the activation TBK1 by recruiting TRAF3-TBK1 complexes

to the mitochondrion. This results in the phosphorylation of IRF3 and the induction of type 1 interferons (IFNs) (Fitzgerald et al. 2003; Gleason et al. 2011; Ma et al. 2012; Paz et al. 2006; Pomerantz & Baltimore 1999). Additionally RIG-I sensing also results in the activation of NF-kB pathway which is a common downstream relay in many different innate immune signalling transductions (Loo & Gale 2011). Its biochemical detection and quantification is important to activation of innate immunity (Noursadeghi et al. 2008). NF-kB is present either as a homodimeric complex of p65 (relA) or as a heteromeric complex of p65 and p50 (Lenardo & Baltimore 1989). Masking of the NF-kB nuclear localization sequence by IkB prevents the entry of NF-kB through the nuclear pore complex. NF-kB dimers are released upon phosphorylation of IkB resulting in nuclear localization of p65 (relA) and unmasking of DNA binding domains, while phosphorylated ikB undergoes proteasomal degradation (Ghosh et al. 1998). It is well known that phosphorylated IRF3 binds IFN-stimulated response elements (ISREs) resulting the induction of additional antiviral genes (Paz et al. 2006; Vasquez et al. 2005; Lazear et al. 2013; Panne et al. 2007) and the secretion of Interferon alpha/beta (IFN-α/β). Binding of IFN-α/β to IFNARs would lead to the activation of downstream JAK (Janus kinase)-STAT (signal transducer and activator of transcription factor) signalling pathways, thereby resulting in the transcription of antiviral IFN-stimulated genes (ISGs).

We studied the antiviral activity of chemically synthesized non-modified, blunt-ended, double-stranded microRNA like molecules known as miRNA mimics. Upon testing different types of commercially available mimics of miR-29b-1* and miR-27b* in tissue culture infection assays, we found that only blunt-ended, non-modified forms were successful in inhibiting HAdV-C5 infection, thus eliminating the miRNA-like activity of these mimics as the basis of their antiviral effect. Importantly, the blunt-ended dsRNA miR-29b-1* and miR-27b* mimics in a sequence-dependent manner suppressed HAdV-C5 infection, since randomized mutant form (mimics with the same nucleotide composition as the host miR-29b-1* and miR-27b* but completely randomized from 5'-3' end) did not exert anti-HAdV-C5 action. Considering that these mimics represented a form of short dsRNA molecules, we probed for the mechanism by which these mimics inhibited the infection. Results obtained from the siRNA-mediated knockdown of dsRNA sensors clearly suggested that RIG-I activated either directly or indirectly by these mimics was required for the induction of the antiviral response. These mimics also exhibited broad-spectrum antiviral activity since they not only suppressed infection of HAdV-C5, but also IAV, HRV1A, SFV and VSV infections.

Results

miR-29b-1* and miR-27b* mimics inhibit HAdV-C5 infection

We carried out gain of function studies to evaluate the effect of miR-29b-1* and miR-27b* miRNA mimics in adenovirus infection using different cell lines (A549, T98G, WI38 and HCE). Synthetic blunt-ended miR-29b-1*and miR-27b* mimics (obtained from Qiagen) were transfected into these cells, followed by infection with wild type HAdV-C5 and the infection index (ratio of infected cells over total number of cells) was assayed 24h p.i. The infection dose used gave about 50% infected cells in the negative (non-transfected) control. After infection, the cells were fixed and immunostained for the late viral protein VI. Along with the wild type mimics (miR-29b-1* and miR-27b*), seed mutant and randomized mutant miRNA mimics were used as controls (**Figure 1A**). miR-29b-1*Seed (2-4) mutant and miR-27b*Seed (2-4) mutant mimics were designed such that 2nd, 3rd and the 4th nucleotide from the 5' end was mutated, but the overall base composition of the seed region was maintained. For miR-29b-1*Seed (4,6) mutant, base composition was not preserved and mutations were introduced in the 4th and 6th nucleotide from the 5'end. The sequences of miR-29b-1*Randmutant and miR-27b*Randmutant mimics were randomized completely compared to their native (wild type) sequences while maintaining their base composition. The sequence information of the mimics used is provided in **(Supplementary Table S1)**. We observed that miR29b-1*, miR-27b*, miR-29b-1*Seed mutant and miR-27b*Seed mutant mimics significantly inhibited the infection of A549, T98G and HCE cells at a concentration of 10nM (**Figure 1B**). In case of WI38 cells, 0.5nM of the mimics was sufficient to inhibit the infection. The levels of E1A protein were significantly reduced in the miR-29b-1* and miR-27b* transfected A549 cells compared to the cells transfected with scrambled control - siAllstar (**Figure 1C**). E1A is the first adenoviral gene to be transcribed. E1A was used to test at what step the infection was affected. The results suggested that the effect is probably at an early stage of infection.

Only blunt-ended and unmodified mimics of miR-29b-1* and miR-27b* inhibit the infection

miRNA mimics obtained from different commercial vendors can have different effects upon transfection due to the changes in synthesis batch, chemical modification to one of the strands and the structure of the mimic. This could result in undesired side-effects such as passenger strand loading. Hence it is considered as a good practice to test mimics from different commercial vendors and check for the uniform effects. For this purpose, we treated A549 cells with different types of mimics obtained from Qiagen, Dharmacon and Microsynth. Similar to naturally occurring cellular

miRNAs, all the commercially available mimics consist of a guide and a passenger strand (**Figure 2A**). The infection experiment presented in **figure 1B**, used Qiagen mimics. Since both the guide and the passenger strand in these mimics were left unmodified, theoretically either of the strands could be loaded into RISC. The passenger stand is chemically modified in Dharmacon mimic in order to make the guide strand to become preferentially loaded into RISC complex. We additionally tested modified Dharmacon mimics where the guide strand only was modified thereby keeping the passenger strand active (Dharmacon Swop mimics). The purpose was to understand if the inhibition of infection was mediated by the passenger strand getting loaded into the RISC complex. Both the guide and the passenger strands from Dharmacon mimic and Dharmacon swop mimic had overhangs while the overall mimic from Qiagen was blunt-ended. We also treated the cells with two types of mimics from Microsynth, one of which was similar to the Qiagen mimics (i.e. blunt-ended and unmodified) and the other which contained two nucleotide 'UU' 3'-overhangs in both the strands (**Figure 2A**). Transfection with the above-mentioned mimics and infections with HAdV-C5 were carried out in the same way as described above. Additionally, cells were also treated with the Dharmacon mimic and Dharmacon Mimic swop together in equal ratios (Dharmacon combined) to test if the combination was responsible for the infection reduction phenotype. Results showed that only blunt-ended, non-modified mimics of miR-29b-1* and miR-27b* from Qiagen and Microsynth were successful in inhibiting the infection **(Figure 2B)**. The presence of an overhang, or any modification completely reversed the phenotype. Taken together, these results strongly suggest that the infection phenotype caused by the Qiagen mimics is not due to these mimics functioning as miRNAs.

miR27b* and miR29b-1* mimics induce a vigorous type I interferon response

To check if the transfected miR-29b-1* and miR-27b* mimics in the form of short dsRNA could lead to induction type I interferon response, and perhaps explain the antiviral effect of these mimics. We next probed whether the Qiagen miR-29b-1* and miR27b* mimics induced type I interferon response, and thus caused inhibition of infection. For this purpose, we transfected A549 and WI38 cells with the mimics and collected the mediium 24h, 48h and 60h post transfection. The clarified culture medium was then titered on 293T reporter cells transfected with a reporter plasmid encoding FF-Luc under the control of interferon α/β-inducible MxA promoter, and a plasmid encoding Renilla luciferase under the control of the constitutive SV40 promoter for normalization of transfection efficiencies. Recombinant IFNα was used as a positive control. We observed enhanced FF-Luc activity in cells treated with the growth medium from the miR-29b-1*, miR-27b* and seed mutant mimic tran

-sfected A549 cells (**Figure 3A)**. The medium from WI38 cells transfected with these three mimics induced about two - four fold higher FF-Luc activity than the corresponding A549 medium **(Figure 3B)**. In contrast, essentially no IFN response was observed in A549 and WI38 cells transfected with the miR-29b-1*-Rand mutant or the miR-27b*-Rand mutant. Induction of IFN response correlates with the ability of the mimics to inhibit infection

miR-29b-1* and miR-27b* mimics exhibit broad-spectrum antiviral activity against several viruses

As shown in Fig. 2C, blunt-ended miR-29b-1* and miR-27b* mimics significantly inhibited HAdV-C5 infection in different cells. To probe whether these mimics have broader anti-virus effects, the inhibition of human influenza A virus (IAV- H3N2[X31 strain]), human rhinovirus (HRV1A), Semliki forest virus (SFV) and vesicular stomatitis virus (VSV) by these mimics was investigated. A549 cells were transfected with miRNA and control mimics (randomized mutants) and infected with the above-mentioned viruses for 12h, 20h, 24h and 24h respectively. We found that miR-29b-1*, miR-27b* and the seed mutant mimics inhibited the infection of IAV and HRV1A >100-fold ($p < 0.05$, using Dunn's-test for multiple comparison), SFV > 800-fold ($p < 0.05$) and VSV-GFP >10-fold ($p < 0.05$) **(Figure 4)**.

Activation of RIG-I by miR29b-1* and miR-27b* induces antiviral state in A549 cells

miR29b-1* and miR-27b* mimics (less than 25 nucleotides each) represent a form of short double stranded RNA (dsRNA). dsRNAs are important mediators of IFN induction particularly in response to virus infection. Many studies have previously reported the activation of innate antiviral immune response by dsRNA molecules (Wang et al. 2016; Gantier & Williams 2007; Creagh & O'Neill 2006; Marques et al. 2006). We therefore speculated that the activation of dsRNA sensors such as RIG-I, MDA-5, or TLRs by miR-29b-1*, miR-27b* and the Seed mutant mimics might mediate antiviral functions and protect cells from virus infection. To check if reduction in infection was mediated by the activation of dsRNA sensors, we carried out double transfection experiments **(Figure 5A)**. In these experiments, we reduced the mRNA levels of MDA-5, RIG-I, MAVS, TRIF, TLR3, IRF3, IFNAR2, and MyD88 individually by transfecting A549 cells with their respective siRNAs (siMDA5, siRIG-I, siMAVs, siTRIF, siTLR3, siIRF3, siIFNAR2, and siMyD88). This was followed by a second-round of transfection which was a co-transfection of siRNAs and miRNA mimic or control mimics. Cells were infected with HAdV-C5 or IAV (X31 [H3N2] strain) 24h post second transfection and scored for infection index 24h or 12h hours post infection respectively. We observed no significant inhibition of HAdV-C5 infection

with miR-29b-1*, miR-27b* and seed mutant mimics in cells treated with siRIG-I, siMAVS, siIRF3 or siIFNAR2. In contrast, siMDA-5, siTRIF, siTLR3 and siMyD88 did not significantly reduce the inhibitory effects of the miRNA mimics (**Figure 5B-5I, left panel**). q-RT-PCR was used to validate effective knockdown of the siRNA targets (**Figure 5B-5I, right panel**). Also in IAV infected A549 cells, miR-29b-1* and miR-27b* mimics were no longer able to significantly reduce the infection if intracellular levels of RIG-1 were downregulated, whereas siMDA5 had essentially no effect **(Figure S1)**. These data suggest that miRNA mimics of 29b-1*, 27b* and the seed mutants act as a broad-spectrum viral antagonist through direct or indirect activation of RIG-I and its downstream effectors MAVS, IRF3 and IFNAR2.

Transfection of blunt-ended miR29b-1* and miR27b* mimics results in NFkB activation

It is known that the activation of dsRNA sensors by dsRNA results in the activation of several signalling pathways, one of them being IkB phosphorylation which leads to NFkB activation (Koh et al. 2000)(Zamanian-Daryoush et al. 2000). Activation of the NF-kB pathway is a common downstream event of the innate immune induction. Its biochemical detection and quantification is used to study innate immune cellular activation(Noursadeghi et al. 2008). We studied NF-kB activation upon miRNA mimic transfection of A549 cells by assaying nuclear accumulation of RelA subunit (p65) of NF-kB. Nuclear to cytoplasmic ratios of p65 were measured for all the cells transfected by different miRNA mimics for 12h and 24h. As positive control, we used cells stimulated with varying doses of Poly(I:C) transfection for 6hrs. There was an expected increase in nuclear : cytoplasmic ratio across the Poly (I:C) dose range. Results showed that miR-29b-1*, miR-27b* and the Seed mutant mimic transfections also resulted in significant increase in the ratios compared to the randomized mutant mimic transfections **(Figure 6A)**. To test whether activation of NF-kB contributed to the antiviral effects of miR-29b-1* and miR-27b* mimics, we assayed the effects of the mimics in A549 cells treated with IkB (IKK-2) inhibitor, TPCA-1(Podolin et al. 2005). As expected miR-29b-1* and miR27b* reduced HAdV-C5 infection in the absence of TPCA-1. However, in the presence of 2uM TPCA-1, the inhibitory effect of miR-29b-1* and miR-27b* mimics was significantly reduced in A549 cells **(Figure 6B)**.

Discussion

Mode of action of endogenous miRNAs are often studied in the form of transient transfections with synthetic miRNA-like molecules known as miRNA mimics. Major question however is whether these mimics act as functional equivalents to the endogenous miRNAs. A recent study by Jin et al. 2015 reported the accumulation of high molecular weight RNA species and high induction of mature miRNAs (upto 100-fold increase) when these mimics were transiently transfected to the cells. Additionally, these mimics upon transfection, are known to cause non-specific regulatory effects on host gene expression. miRNA mimics when efficiently delivered into the cultured mammalian cells via transient transfection, can bypass the maturation and processing steps of endogenous miRNA biogenesis (reviewed by Jin et al. 2015). These mimics are considered to be fast and economical way to understand the functional relevance of the endogenous miRNAs. Most of the times as in our case, the formulations or the chemical modifications on these mimics are unknown to the users. Such modifications are mainly used to overcome problems like induction of innate immune sensing and RNA degradation (Git 2012). Due to such modifications, the possibility of performing misleading experiments increases significantly. In the first set of experiments performed in this study we observed the anti-adenoviral activity of Qiagen miR-29b-1* and miR-27b* mimics on different cultured cells. Since the mimics with the mutated seed sequences also inhibited HAdV-C5 infection significantly (**Figure 1C**), results from randomized mutant mimics suggest that sequences outside the seed are behind the inhibitory effect.

In our study, we did not observe significant inhibition of viral infection in all miR-29b-1* and miR-27b* mimics. Only blunt ended, non-modified mimics of miR-29b-1* and miR-27b* obtained from Qiagen and Microsynth gave rise to anti-adenoviral effects (**Figure 2A and 2B**). These results clearly suggested that these mimics probably did not function as endogenous miRNAs. The commercially available miRNA mimic consists of two completely complementary strands. However, an endogenous miRNA consists of a guide and passenger strand, which partly lack complementarity between each other. In certain circumstances, the complimentary strand of miRNA mimic, instead of the desired strand might get unintentionally loaded into the RISC complex. When this happens, the result would not be a true representation of the original situation. Such a situation could arise with the Qiagen mimics of miR-29b-1* and miR-27b* where both the strands was left unmodified and completely complementary to each other. To address this problem, we ordered several versions of the same mi

-mic from Dharmacon (explained below) and used them in infection assay: 1) miRNA mimic with active guide strand and an and an inactive or modified passenger strand (normal Dharmacon mimic), 2) miRNA mimic with active passenger strand and an inactive guide strand (swopped Dharmacon mimic). Additionally, above mentioned versions of Dharmacon mimics were co-transfected (combined Dharmacon mimic) together to check if the antiviral effect was due to the combined effect of both guide and passenger strand getting loaded into the RISC complex or due to individual effect of either strands (**Figure 2A and 2B**). The major outcome from this experiment was that none of the Dharmacon mimics successful in inhibiting the infection. This clearly suggested that antiviral effect of blunt-ended, non-modified mimics of miR-29b-1* and miR-27b* was not due to the passenger strand or the combined effect of both the strands of the mimic.

dsRNAs are well known inducers of innate immune response and IFN genes (reviewed by Karpala et al. 2005; Gantier & Williams 2007; Gantier & Williams 2010; Caskey et al. 2011). Since Qiagen miR-29b-1* and miR-27b* mimics represent a form of short dsRNA, we hypothesized that the transfection of these mimics to the cultured cells would result in the induction of type I interferon response and thereby explain the anti-adenoviral effect generated by these mimics. To address this, we devised an assay to quantitatively measure type 1 interferon, based on dual luciferase expression system (**described in the 'experimental procedures' section**) (Jorns et al. 2006) under the control of an IFN sensitive and constitutive promoter, respectively (**Figure 3A and 3B**) and found that type I IFNs were produced upon transfection of blunt ended, non-modified mimics of miR27b* or miR29b-1*. This also suggested that these short dsRNA mimics could directly or indirectly induce innate immune sensing in the cells and thereby exhibit broad spectrum antiviral activity. This was confirmed by the significant inhibition of other viruses such as IAV, HRV 1A, SFV and VSV (**Figure 4**). From the beginning of siRNA technology, it has been clear that particular dsRNA molecules can induce innate immune responses in a sequence-dependent manner (Judge et al. 2005; Saito et al. 2008). One sequence motif indicated in these early studies was 5'-UGUGU-3'. Based on the results obtained from the knockout studies in mice, the immune-stimulatory synthetic siRNAs have been suggested to be sensed by TLR3 and TLR7, and selective 2-O-methyl modification of one strand has been shown to reduce the innate immune responses to these siRNAs (Morrissey et al. 2005; Judge et al. 2006; Judge et al. 2009). Furthermore, Marques et al. 2006 demonstrated that blunt-ended double-stranded siRNAs of 21-27nucleotides can be sensed by RIG-I and this leads to innate immune responses, but this study left open the question of sequence-specificity of the RIG-I activation. These early studies considered the immunostimulatory

function of siRNAs as an undesired side effect which had to be suppressed, but more recent studies have highlighted the potential of dsRNA sensor stimulation as a broad-range antiviral therapeutic. For example, a 99nt long 5'ppp M8 dsRNA molecule designed for optimal RIG-I activation was demonstrated to suppress influenza infection in mice (Chiang et al. 2015), and expression of the EMCV RNA-dependent RNA polymerase (RdRP) in mice was demonstrated to induce lifelong elevation of ISGs in a MDA5- and MAVS-dependent manner, and thus potentially to create a broad antiviral defence for the mice (Painter et al. 2015). The EMCV RdRP achieved the activation of MDA5 by increasing endogenous dsRNA levels (identified by anti-dsRNA staining) in mouse tissues. Intriguingly, the elevated ISG levels did not exert any obvious adversary effects on the health of the mice. With this regard, we carried out siRNA mediated knockdown of several key dsRNA sensors and its effector molecules (**Figure 5A-I**) and found that these short blunt-ended, non-modified dsRNA mimics of miR-29b-1* and miR-27b* either directly or indirectly activated RIG-I (**Figure 5B**) and this probably gave rise to broad antiviral response.

The optimal ligand for RIG-I is considered to be a RNA with a free 5' triphosphate (5'ppp) end followed by a short (at least 19bp) dsRNA region (Schlee et al. 2009). However, short dsRNAs (at least 25nt) having 5' monophosphate or diphosphates can also be recognized by RIG-I (Marques et al. 2006; Takahasi et al. 2008; Judge et al. 2005; Gantier & Williams 2010). The 5'ppp is recognized by the CTD (carboxy terminal domains) domain of RIG-I, whereas the helicase domain is known to interact with the dsRNA. It is not clear how much sequence specificity is in RIG-I ligand recognition. The crystal structure of RIG-I helicase and CTD domain with a 5'ppp dsRNA ligand indicated that most of the contacts are with the sugar phosphate backbone of both RNA strands (Jiang et al. 2011). However, literature has examples that would suggest that sometimes ligand recognition by RIG-I also involves sequence specificity. For example, RIG-I has been described to "sense" specific viral sequences: a poly-U/UC sequence (100nt in length) in the 3' untranslated region of the HCV genome acts as a RIG-I ligand in virus infected cells (Saito et al. 2008; Schnell et al. 2012; Uzri & Gehrke 2009) and the stable stem-loop structure (bases 70-114) on the positive strand of Sendai virus defective viral genomes is another example of a sequence-specific RIG-I ligand (Xu et al. 2015). However, in both of these cases the sequence specificity might, at least in part, come from the necessity to build a certain RNA secondary structure. There are occasional reports that, similar to our results, also rather short blunt-end dsRNA sequences without 5'ppp can elicit innate immune response in a RIG-I-dependent manner (Marques et al. 2006; Takahasi et al. 2008). So, we speculate that the possible exp

-lanation for the apparent sequence-specific, RIG-I-dependent innate response from the blunt ended, non-modified mimics of miR-27b*and miR-29b-1* could arise from the specific 'UGGU' motif found in these mimics along with miR-29b-1*(2-4) SeedMutant and miR-27b*(2-4) SeedMutant mimics (**Figure S2**). Since only these mimics led to strong inhibition of viral infection, it would be a good idea to map the activating sequence more carefully by further characterizing the mid part of the non-seed region of these mimics (i.e. mutate this region of the mimic and check for its effect upon transfection).

It would be important to establish whether these blunt ended, non-modified mimics are directly recognized by RIG-I. In our first attempts to solve this question we tried to assay in vitro (as described by Marques et al. 2006) whether the miRNA mimics activate the ATPase activity of RIG-I. The assays have so far been inconclusive since our positive control, a blunt-end 27bp dsRNA sequence (Marques et al. 2006), that has previously been described to activate RIG-I, did not work either. One possible explanation is that our purified RIG-I was inactive. To control whether the RIG-I was the problem, small amounts of functionally active RIG-I could be retested in our assay. Another possible explanation is that the positive control dsRNA, and our miR-29b-1* and miR-27b* mimics, have wrong 5' structures (both the Qiagen mimics have 5' OH). Marques et al. 2006 did not specify the 5' structure of the dsRNAs used, but the RNAs were from Dharmacon, and they might have had 5' monophosphate at least on one of the strands. When transfected into cells, our miR-29b-1* and miR-27b* mimics are expected to become 5' monophosphorylated (Nykänen et al. 2001) by Clp1 (Weitzer & Martinez 2007). Hence, we would need to phosphorylate the 5' ends of our miRNA mimics and the positive control dsRNA for use in the RIG-I in vitro ATPase assay.

In general, one has to be a bit careful when interpreting the *in-vitro* assay results, since the assay conditions might not exactly mirror *in-vivo* conditions very well (for example, with respect to salt concentrations or concentrations of RIG-I or the putative ligands). A possible alternative for the in vitro assay would be the expression of tagged RIG-I in cells, affinity-purification of RIG-I and determination of co-purified RNA molecules by next generation sequencing (Sanchez David et al. 2016), provided that the sequencing can be tuned to such short sequences as our miRNA mimics. It would be interesting to check if the differential effects of miR-29b-1* and miR-27b* mimics could be due to different stabilities of the transfected mimics in cells. As mentioned above, the mimics are expected to be 5' monophosphorylated by Clp1 after entering the cytoplasm and this could affect the stability of the mimics, but at least the 5' nucleotide should not affect the phosphorylation efficiency (Weitzer & Martinez 2007). However, Chemical modifications generally absent in the endogenous mi

-RNAs (Wang 2011; Thomson et al. 2013) and nucleotide changes in the strands complementary to the miRNA strands of interest (Lim et al. 2005; Garcia et al. 2011) are mostly introduced to the mimics to make them more stable. Additionally, RNA stability can also be increased by the addition of 2'-O-fluorine or 2'-O-methyl groups to the 2'OH of the sugar ring (Behlke 2008).

Reduced cell numbers in miR-29b-1* and miR-27b* mimic and SeedMutant mimic transfected cells was suggestive of the toxic effect of these mimics. The RIG-I mediated signaling needs to tightly regulated and also needs breaks, since toxicity is associated with RIG-I activation (Ireton & Gale 2011). For a long time, the toxicity effects were thought to be due to heightened interferon response, since this response upregulates pro-apoptotic genes. However, RIG-I signaling was recently shown to activate apoptosis more directly as well, via MAVS-TRAF3-TBK1-IRF3 and through interaction of IRF3 with BAX (Chattopadhyay et al. 2010). In contrast to IFN induction, the apoptosis pathway required TRAF2 and TRAF6 as well, but not the transcriptional activity of IRF3. In addition to the antiviral effects of the blunt ended, non-modified mimics of miR-29b-1* and miR-27b*, it is also important to consider the toxicity effects associated with these mimics.

RIG-I could also be indirectly involved in responses elicited by these miR-29b-1* and miR-27b* mimics. RIG-I activity is regulated at multiple levels: by posttranscriptional modifications and by interaction partners (reviewed by Chan & Gack 2015;Fullam & Schröder 2013). It is possible that these miRNA mimics are not directly sensed by RIG-I, but by another dsRNA sensor that works upstream of RIG-I, or that our mimics bind to a negative regulator of RIG-I and "neutralize" this regulator which in turn leads to upregulated RIG-I activity, possibly against endogenous ligands. One possible approach for these scenarios would be to use siRNA-mediated knockdown of known RIG-I dsRNA binding protein interaction partners and assess the effect of the knockdown on our miRNA mimics' action. We could test a following list of genes (from Fullam & Schröder 2013): LGP2, DDX3, DHX9, DDX60, OAS-proteins/RNAseL, PACT and ZAPS. LGP2 binds to dsRNA but has no specificity for 5'ppp (Li et al. 2009). It was Initially described as an inhibitor of RIG-I (but an activator of MDA-5), but can also be an activator of RIG-I, at least in the context of RIG-I ligands generated by cytoplasmic RNA polymerase III (Satoh et al. 2010). DDX3 can be involved in signalling pathways leading to IFNβ production (Soulat et al. 2008; Schröder et al. 2008; Oshiumi et al. 2010), but there is no direct evidence to suggest that it acts as a RNA sensor; only one study suggested this because an interaction between DDX3 and MAVS was detected in an IP assay (Oshiumi et al. 2010). DHX9 serves as a potential dsRNA sensor in myeloid dendritic cells (Zhang et al. 2011). Poly I:C was used in the study,

but there was no direct evidence to suggest that it was involved in RNA binding. DDX60 serves as a Cofactor for detection of dsRNA, functions in RLH-mediated signalling together with RIG-I or MDA-5 (Miyashita et al. 2011). dsRNAs stimulate OAS1, OAS2 and OAS3 and this leads to production of 2'-5' oligoA and activation of RNaseL (Choi et al. 2015). Activated RNaseL in turn can create ligands for RIG-I (Malathi et al. 2007). PACT (protein activator of PKR) binds C-terminal repressor domain of RIG-I and potentiates RIG-I mediated signalling (Kok et al. 2011). However, since PACT is also involved in miRNA processing and RISC loading (Heyam et al. 2015), RNAi-mediated approaches for PACT silencing would not necessarily work, and hence CRISPR-Cas9 mediated gene silencing would be required. ZAPS (Zinc-finger antiviral protein shorter isoform) has been shown to potentiate RIG-I oligomerization and ATPase activity upon ligand binding, as well as RIG-I-MAVS mediated innate immune responses in cells (Hayakawa et al. 2011).

In addition, also the effect of knockdown of PKR and Clp1, as well as that of TRAF2, TRAF3 and TRAF6 could be tested. Furthermore, if any of the above mentioned DExD/H-box RNA helicases turns out to be "involved" in the action of our miRNA mimics, it would be an interesting observation as many of the DExD/H-box RNA helicases have functions also in transcription and/or RNA metabolism, including mRNA processing, and thus their apparent involvement might be also indirect.

As a major outcome, our study found a unique way by which two double stranded, non-modified and blunt ended miRNA mimics act as broad range antiviral molecules. Based on the results obtained from the above studies, we believe that the gain-of-function studies employing transient transfections with miRNA mimic should be carried out with caution since these molecules may not necessarily mimic the effect of the endogenous miRNA. Above results suggested that the antiviral activity could be induced by sequence specific activation of RIG-I by these mimics. However, the exact mechanism of the RIG-I activation by these mimics require further investigations.

Experimental procedures

Cell Lines and Viruses

A549 human lung epithelial carcinoma cells, WI38 human embryonic lung fibroblast cells, T98G human glioblastoma multiforme tumor cells, HCE Human Corneal epithelial cells and 293T human embryonic kidney cells expressing the SV40 large T antigen and E1 region of HAdV-C5 were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal calf serum (FCS, In

-vitrogen) at 37^0c under 5% CO_2 and 98% humidity. Cells were passaged every 2-4 days with dilution of 1:2 to 1:20 and used at passage 1-25 for experiments. HAdV-C5 was grown in A549 cells as described previously (Greber et al. 1996; Greber et al. 1993). HRV1A was grown as described before(Jurgeit et al. 2010). Other viruses were kindly provided by Yohei Yamauchi (University of Bristol).

miRNA mimics used in the transfections

Blunt-ended, non-modified miRNA mimics and controls were purchased from Qiagen or Microsynth. miRNA mimics with overhangs from Dharmacon or Microsynth were also used for transfections. Additionally, swopped miRNA mimics were obtained from Dharmacon. Working stocks of 2µM were made for each miRNA and stored together with the 20µM stocks at -80°C. details of the mimics used in this work are listed in the **Supplementary Table S1**

miRNA transfections and imaging

To test the antiviral activity of miR-29b-1* and miR-27b* mimics, reverse transfection experiments were carried out in 96-well plate format using established protocols (ThermoFisher Scientific protocols). All plates contained negative controls such as MOCK (no miRNA mimic), siAllstar (non-targeting siRNA), randomized mutant mimics (miR-29b-1*RandMutant and miR-27b*RandMutant). Lipofectamine RNAiMAX (0.15µl, Invitrogen) was mixed with 9.85µl of OptiMEM reduced serum medium (Gibco) and added to a well containing 100 nM miRNA mimic diluted in 10µl OptiMEM. The plates were incubated at room temperature (RT) for 1 h, and thereafter 8000 (A549, T98G and HCE) or 10000 (WI38) cells were added to each well in a volume of 80µl DMEM supplemented with 16% FCS, resulting in a final FCS concentration of 12.8%. Plates were incubated at 37°C for 24h. Cells were infected with different viruses individually: HAdV-C5, H3N2 (X-31), HRV1A, SFV and VSV-GFP. The dosage of virus infection was dependent on different cell types used for infection. The infection dose used was aimed at getting about ~50% (HAdV-C5 in A549 [24h]), ~25% (HAdV-C5 in T98G[24h]), ~35% (HAdV-C5 in WI38[24h]), ~12% (HAdV-C5 in HCE[24h]), ~40% (IAV- H3N2[X31 strain] in A549[12h]), ~9% (HRV1A in A549[20h]), ~8% (SFV in A549[24h]) and ~8% (VSV-GFP in A549[24h]) infected cells, in the negative (non-transfected) control. Post infection, cells were fixed with 4% PFA (paraformaldehyde), followed by quenching with 25mM NH4Cl in 1X phosphate buffered saline (PBS). The cells were subsequently permeabilised with 0.5% Triton (TX-100) in 1X PBS for 5 min at RT. In the case of HAdV-C5_wt infection, cells were immunostained with primary rabbit anti-protein VI antibody

(Burckhardt et al. 2011). For X-31 (H3N2), nucleoprotein (NP) and Hemagglutinin (HA) were immunostained with primary ''HB-65'' mouse monoclonal antibody supernatant from the hybridoma cells and anti-X31 rabbit polyclonal antibody (Pinda) respectively. For HRV1A infected cells, viral protein (VP2) was immunostained with monoclonal mouse IgG purified from hybridoma clone R16-7 and nsp3 protein from SFV infected cells was immunostained using nsP3-specific antibodies (Peränen & Kääriäinen, 1991, Salonen et al., 2003). This was followed by the incubation with specific *secondary anti-mouse or anti-rabbit antibodies conjugated* to Alexa Fluor 488 (Life Technologies) for 1hr at RT. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) in 10% Goat Serum (GS). All the primary, and secondary antibodies used were diluted in 10% GS. After staining, plates were imaged with the Image Xpress Micro XL high throughput microscope (Molecular Devices) using 10X or 20X S Fluor objective with a 0.45 numerical aperture, 9 (3×3 grid) or 16 (4×4 grid) with no spacing and no overlap per well. DAPI stain was used to mark the cell nucleus, and a custom-made script (Matlab; Mathworks, USA) or a custom-made CellProfiler (version 2.0) pipeline was used to quantify the average nuclear intensity of the protein VI of HAdV-C5, cytoplasmic intensity of VP2 protein of HRV1A, nsp3 protein of SFV and GFP intensities of VSV-GFP virus to measure the infection index (http://cellprofiler.org)(Carpenter et al. 2006). The pipeline or the script will be made available upon request).

Western blot analysis of E1A expression

A549 cells grown in 6-well plates were reverse transfected for 9h or 24h with Qiagen miRNA mimics and control mimics as described above. Lysates were prepared by scraping cells (approximately 7.2 x 10^5) in 1X SDS sample lysis buffer (25% (w/v) 4X SDS page buffer [200 mM tris-Cl (pH 6.8), 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol], 4% SDS, 50mM DTT and 1mM phenylmethylsulphonyl-fluoride (PMSF)). Lysates were cleared by centrifugation at 13,000 g for 10 min. Protein concentration of the lysates was determined using the BCA protein assay kit (Pierce™ BCA Protein assay kit, catalogue number: 23225) 100ug of cell lysates / lane was separated by SDS page under reducing conditions. After transfer to nitrocellulose (Hybond-C, Amersham, Buckinghamshire, UK), blots were blocked with 5% (w/v) non-fat dry milk in TBST (10mM Tris, 150mM NaCl and 0.05% (v/v) Tween-20, pH 8.0). HAdV-C5 EIA was probed overnight at 4°C with mouse monoclonal (M73) antibody (1:1000) [abcam -ab28305]. Calnexin (housekeeping protein) was detected by rabbit polyclonal Calnexin Antibody (Hc 70:sc11397) [Santa Cruz Biotechnology] (1:100) at 4°C for 2h. All the primary antibodies used were diluted in TBST containing 2.5% non-fat dry milk.

Immunoreactive proteins were visualized using HRP-conjugated IgG for 1h at 4°C in 5% TBST [Goat-anti-mouse for M73 (E1A) and Goat-anti-rabbit for Calnexin] and enhanced chemiluminescence (ECL, Amersham).

Measurement of IFN induction

2.5nM Qiagen miR-29b-1* and miR27b* mimics were reverse transfected in A549 cells in 96-well plate in the same way as described above. The growth medium on which the transfected cells were growing was collected at different time points (24h, 48h and 60h) and titered on 293T reporter cells (80000 cells/well of 24 well plate) transfected overnight with a reporter plasmid encoding Firefly luciferase (pGL3-Mx1-FFLuc) under inducible MX1 promoter (200ng), plasmid encoding Renilla luciferase (pRL-SV40-RenillaLuc) under constitutive SV40 promoter (50ng) and PET28a (750ng) plasmid using 2ul JETPEI in a final volume of 50ul NaCl (150mM). The pasmids were diluted in 150mM NaCl prior to the addtion of JETPEI. As a positive control for IFN induction, some of the transfected 293T cells were treated with different concentrations of recombinant IFNα (1,25U, 2.5U, 5U and 10U). 20h post treatment 293T cells were lysed and Firefly (FF-Luc) and Renilla (REN-Luc) luciferase activities were measured using dual-luciferase reporter assay kit (Promega) as per the manufacturer's instructions.

Double transfection experiment to study the effect of Qiagen miR-29b-1* and miR-27b* mimic activation of dsRNA sensors

There were two rounds of reverse transfections. In the first round, reverse transfection with siRNAs was carried out in 24-well plates. A549(40000 cells /well) were transfected with 20nM siRIG-I, siMDA-5, siTLR3, siTRIF, siMAVs, siIRF3, siMyD88 and siIFNAR2. All the siRNAs used in the transfection were obtained from Dharmacon (siGenome siRNAs). 48h post transfection, cells transfected with the siRNAs in the initial round were detached and subjected to co-transfection in 96-well plate with 20nM siRNAs and 10nM Qiagen miRNA mimic or control mimics. Reverse transfections were performed in the same manner as described above. For transfections in 24-well plate, the transfection volumes were upscaled according to the well area. 24h post the second transfection cells were infected with HAdV-C5 or IAV (X31 [H3N2] strain) and scored for infection index 24h or 12h hours post infection respectively. Immunostaining, Imaging, and Image analysis were carried out in the same manner as described above.

NF-kB translocation assay

A549 cells (8000 cells /well) were reverse transfected with 10nM Qiagen miRNA mimics and control mimics in 96-well plate in the same way as described above. 12h or 24h post transfection, cells were fixed, permeabilised and stained with DAPI and AlexaFluor 647 succinimidyl ester (SuccEst) [ThermoFisher Scientific A20006] dye. Stock solution of SuccEst was 5 mg/ml in DMSO and was used at 1:10000 dilution in 100mM NaHCO3-2.5mM Na2CO3 buffer, 5 minutes staining at RT. p65 (relA) subunit of NF-kB was immunostained with rabbit monoclonal anti-p65 antibody (#8242) (Cell Signaling Technology) and secondary Alexa Fluor 488-conjugated anti-rabbit antibody (Life Technologies). As a positive control cells were transfected with 100nM, 1uM and 10uM concentrations of PolyI:C, 6h prior to fixation. Imaging was carried out in the same way as described above. DAPI stain was used to mark the cell nucleus and SuccEst dye was used to mark the cell boundary. A custom-made CellProfiler (version 2.0) pipeline was used to quantify nuclear : cytoplasmic ratios of p65. A detailed description of the imaging procedures and pipeline will be available on request.

Experiments with IkB (IKK-2) inhibitor TPCA-1

Prior to the transfection of A549 cells with 10nM Qiagen miRNA mimics and control mimics, cells were treated with or without 2uM TPCA-1(cat.no2559) (Tocris Biosciences). 24h post transfection cells were infected with HAdV-C5 in the same way as described above. 24h p.i cells were fixed, immunostained, imaged and analyzed in the same way as described above. In the TPCA-1 treated cells, the drug was present throughout the experiment till the time of fixation.

Figure legends

Figure 1: miRNA mimic-infection assay to study the effect of miR-29b-1* and miR-27b* in the form of mimics. A) graphical representation of miRNA mutant mimics. Top panel shows the design of the Seed mutant where the seed region (shaded in red) is mutated. Bottom panel shows randomized mutant where not only the seed but the entire sequence is randomized. **B)** Effects of 10nM miRNA mimics on HAdV-C5-wt infection in A549 cells (topmost panel), T98G cells (2nd panel), HCE cells (bottommost panel) and 0.5nM mimics in WI38 cells (3rd panel). The cells were infected 24 h post miRNA mimic transfection and analyzed 24h post infection. Shown are infection indices (ratios

of nuclei positive for the viral protein VI over total number of nuclei) and the number of nuclei analyzed. The values represent mean ± SD from three technical replicates and are compared to the controls (miR-29b-1*Randmutant and miR-27b*Randmutant transfected cells) by pairwise comparisons using Dunn's test for multiple comparison. *P < 0.05, **P ≤ 0.01, and ***P<0.001. Statistical significance was computed only for Infection indices. **C)** Western blot analysis of E1A protein levels of HAdV-C5 at 9 h and 24 h p.i. in control and miRNA mimic-transfected A549 cells. Calnexin was used as a loading control.

Figure 2: Effect of different types of miR29b-1* and miR-27b* mimics from different commercial vendors on HAdV-C5 infection. A) Cellular miRNA **(top left panel)** consists of a guide and a passenger strand. The seed region (orange band) in the guide strand extends from 2nd to 8th nucleotide on the 5' end. Qiagen mimic **(bottom left panel)** is a double stranded, non-modified blunt end molecule. Dharmacon mimic **(top middle panel)** and Dharmacon Mimic swop **(bottom middle panel)** are double stranded molecules with overhangs and having a chemically modified passenger strand or guide strand, respectively. These modifications are mainly used to overcome problems like induction of innate immune sensing and RNA degradation and increases the stability of the mimics (Git 2012). Blunt-ended Microsynth mimics **(top right panel)** share an identical sequence, chemistry and structure with the Qiagen mimics. The Microsynth mimic with an overhang (bottom right panel) contains two additional 'UU' nucleotides at 3' end of both the strands. **B)** Effects of 10nM of the above-mentioned miRNA mimics on HAdV-C5-wt infection in A549 cells. Cells were infected 24hrs post transfection and fixed 24hrs post infection. Shown are infection indices (ratios of nuclei positive for the viral protein VI over total number of nuclei) and the number of nuclei analyzed. The values represent mean ± SD from three technical replicates and are compared to the non-transfected cells by pairwise comparisons using Dunn's-test for multiple comparison. *P < 0.05. Statistical significance was computed only for Infection indices.

Figure 3: Tranfection of A549 and WI38 cells with the blunt-ended, non-modified mimics of miR29b-1* and miR27b* induces IFN α/β secretion. A) A549 Cells and **B)** WI38 cells were transfected with the indicated amounts of mimics and the culture medium was collected after the indicated times post transfection. The clarified culture media were titered on indicator 293T cells transfected with plasmids directing the synthesis of Firefly luciferase from the IFN-inducible MXA promoter and Renilla luciferase from the constitutive SV40 promoter. Recombinant interferon alpha was used as a control. After 20h, 293T cells were lysed and Firefly (FF-Luc) and Renilla (REN-Luc) lucif

-erase activities were measured. The FF-Luc activities were normalized to that of REN-Luc. Fold-inductions were calculated by comparing the FF-Luc/Renilla-Luc signal to the untreated cells. Bars represent mean signal ratios ± SD calculated from three technical replicates.

Figure 4: Blunt-ended miR-29b-1*, miR-27b* and Seed mutant mimics confer broad-spectrum antiviral activity to the cells. Antiviral effects of 10nM blunt-ended, non-modified miR-29b-1* and miR27b* mimics on **A)** Human Influenza A virus (X31) – 12hpi, **B)** Human Rhinovirus (HRV1A) - 20hpi **C)** Semliki Forest virus (SFV) - 24hpi and **D)** Vesicular Stomatitis GFP-virus (VSV-GFP) in A549 cells 24h post infection. Prior to infection, cells were transfected with miRNA and control mimics for 24hrs. After Infection, fixed cells were stained for different viral proteins (Nucleoprotein (NP) and Hemagglutinin (HA) for IAV, Capsid protein (VP2) for HRV1A, Non-structural protein 3 (nsp3) for SFV, and GFP signal was used for VSV-GFP). Shown are the ratios of positively infected cells (infection index) and the number of nuclei analyzed. The values represent mean ± SD from three technical replicates. The values were compared to the controls (miR-29b-1*Rand mutant and miR-27b*Rand mutant transfected cells) by pairwise comparisons using Dunn's-test for multiple comparison. *P < 0.05, **P <- 0.01, and ***P<0.001. Statistical significance was computed only for Infection indices.

Figure 5: B**lunt-ended miR-29b-1*, miR27b* induce antiviral state by activating RIG-I. A)** Schematic representation of double transfection experiment. A549 cells were transfected with siRNA targeting dsRNA sensors or their downstream effectors for 48h. This was followed by 24h co-transfection of miRNA/control mimics and siRNAs. Cells were infected 24hrs post second round of transfection and fixed 24hrs post infection. **B-I, left panel)** Shown are the ratios of positively infected cells (infection index) over the total number of cells analyzed. The values represent the mean ± SD from 3 technical replicates. **B)** Results from siRIG-I, **C)** siMDA5, **D)** siTLR3, **E)** siMAVs, **F)** TRIF, **G)** MyD88, **H)** IRF3, **I)** IFNAR2 transfections. The mean values were compared to the controls (siAllstar treated cells transfected with miR-29b-1*Rand mutant and miR-27b*Rand mutant) by pairwise comparisons using Dunn's-test for multiple comparison; *P < 0.05, **P <- 0.01, and ***P<0.001. Statistical significance was computed only for Infection indices. **B-I, right panel) -** q-RT-PCR validation of siRNA-mediated knockdown. mRNA levels were normalized to EEF1A1 and TBP and expressed as mean relative mRNA levels (n=3 per treatment). Bars representing the means ± SD from mock miRNA levels was assessed by one-way ANOVA with a Dunnett post-hoc test; *P <0.05.

Figure 6: Activation of NF-kB by blunt-ended miR-29b-1* and miR-27b* mimics in A549 cells. A) NF-kB nuclear translocation by immunofluorescence imaging (Nuclear: cytoplasmic p65(relA) staining) in miRNA mimic transfected (12hrs- Left panel and 24hrs-Right Panel) or Poly (I:C) stimulated cells. Box and Whisker plots represent median and standard deviation of nuclear:cytoplasmic ratios. Approximately 150 – 300 cells were analyzed per condition. The ratios were compared to the controls (miR-29b-1*Rand mutant and miR-27b*Rand mutant transfected cells). Dunn's multiple comaparison test was used to compute the p-values; *P < 0.05, **P <- 0.01, and ***P<0.001. **B)** Effect of TPCA-1 on miRNA mimic induced NF-kb activation in A549 cells.Cells were transfected with miRNA mimics for 24h with or without TPCA-1(2uM). Cell were then infected with HAdV-C5 for 24hrs, with TPCA-1 still present, fixed and stained for newly synthesized HAdV protein VI. Shown are the ratios of positively infected pVI stained cells (infection index) and the number of nuclei analyzed expressed as the mean ± SD and compared to the controls (untreated cells transfected with miR-29b-1*Rand mutant and miR-27b*Rand mutant) by pairwise comparisons using Dunn's-test for multiple comparison; *P < 0.05, **P <- 0.01, and ***P<0.001. Statistical significance was computed only for Infection indices.

Supplementary information

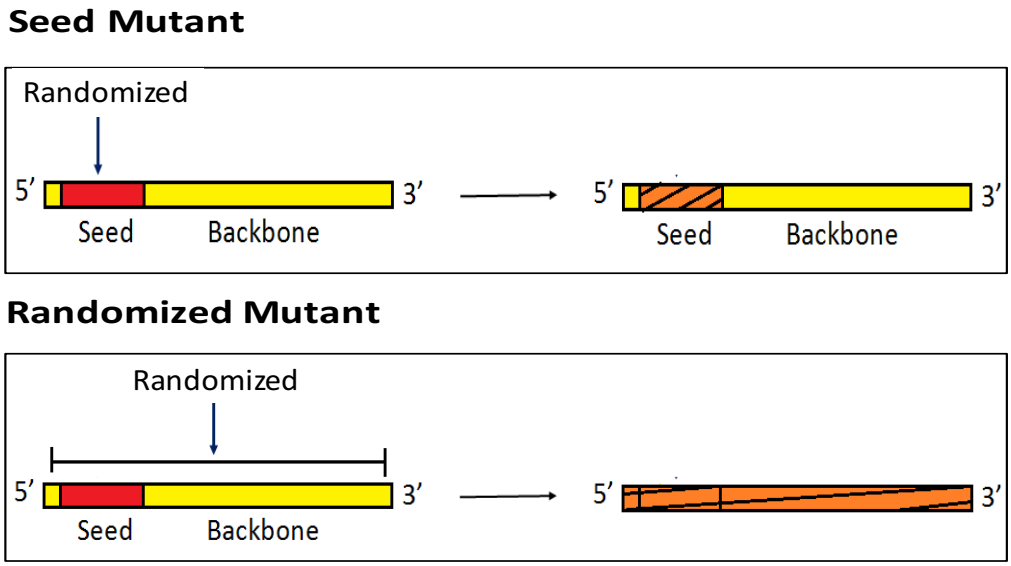
Table S1: list of miRNA mimics and siRNA used in the study

Figure S1: miR-29b-1*, miR27b* lead to the activation RIG-I and significantly inhibit IAV infection. A549 cells were transfected with siRNA targeting MDA5 and RIG-I for 48h. This was followed by 24h co-transfection of miRNA/control mimics and siRNAs. Cells were infected 24hrs post second round of transfection and fixed 12hrs post infection. Shown are the ratios of positively infected cells (infection index) over the total number of cells analyzed. The values represent the mean ± SD from three technical replicates. The mean values were compared to the controls (cells transfected with miR-29b-1*Rand mutant and miR-27b*Rand mutant) by pairwise comparisons using Dunn's-test for multiple comparison; *P < 0.05, **P <- 0.01, and ***P<0.001. Statistical significance was computed only for Infection indices.

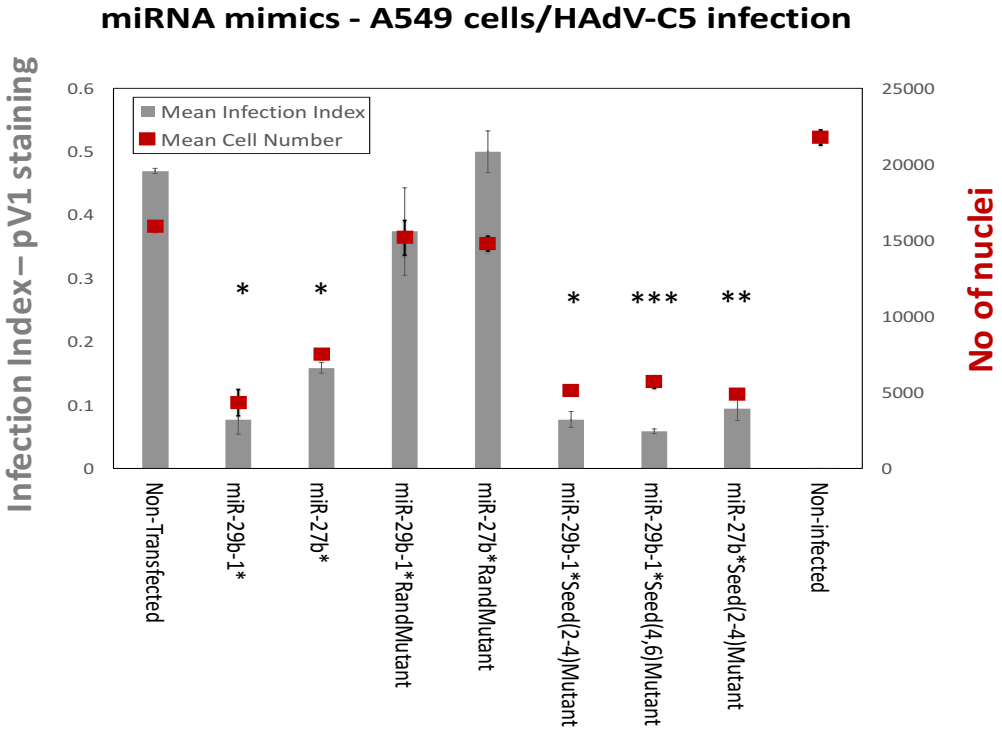
Figures

Figure 1

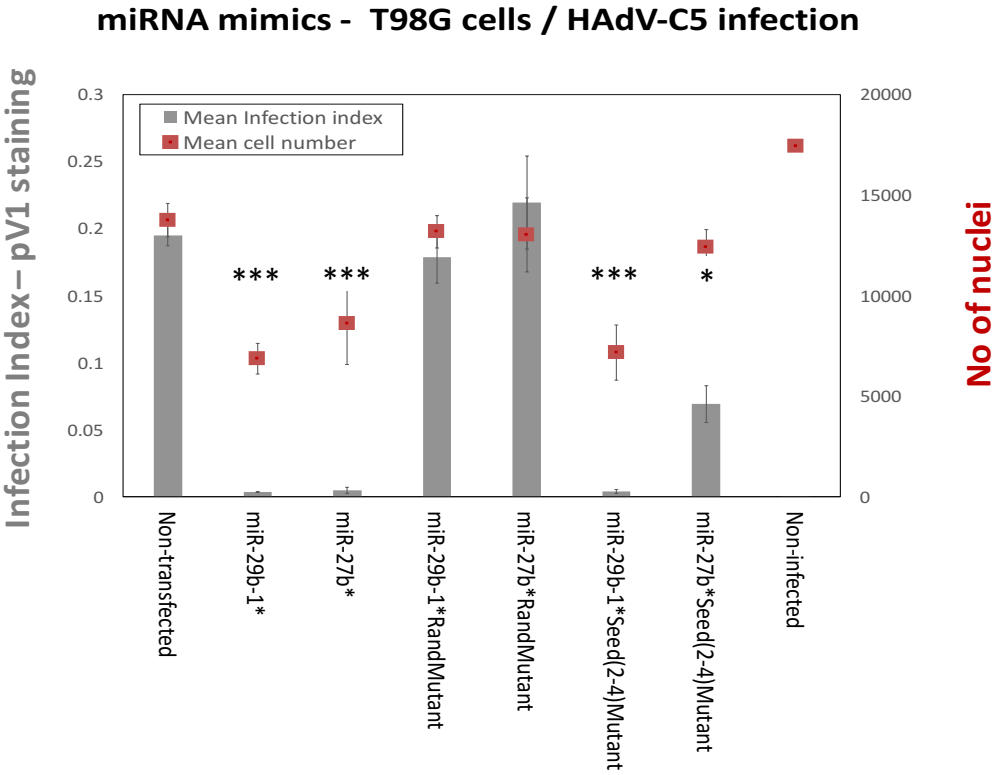
A



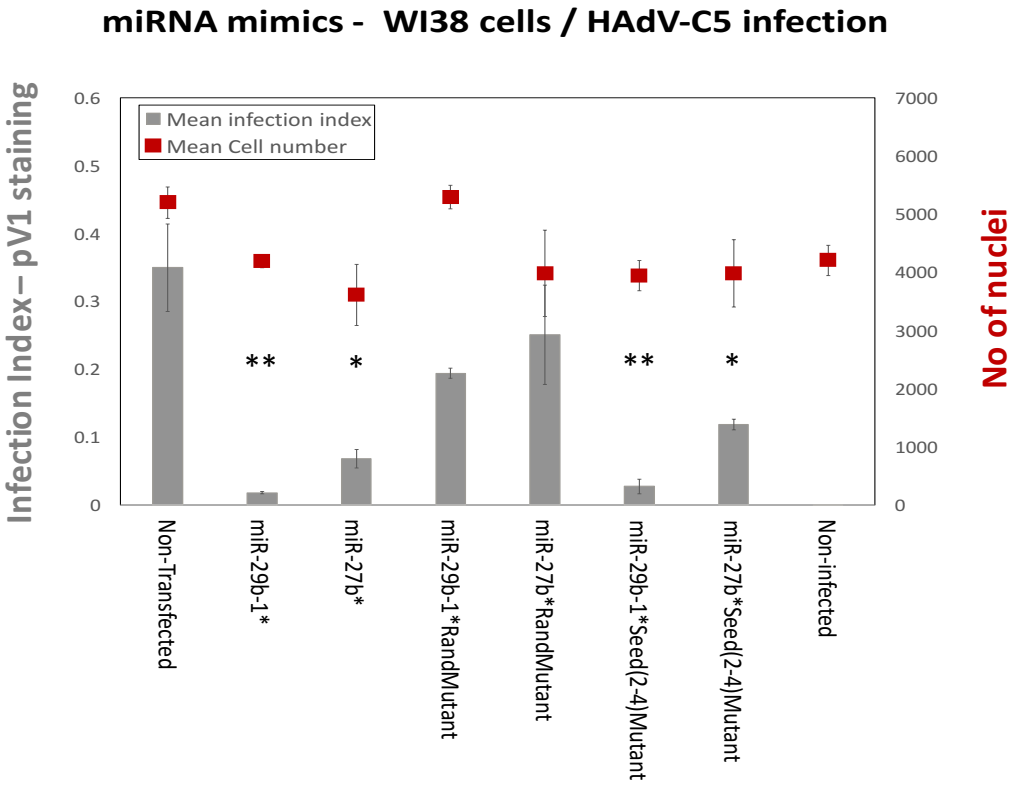
B



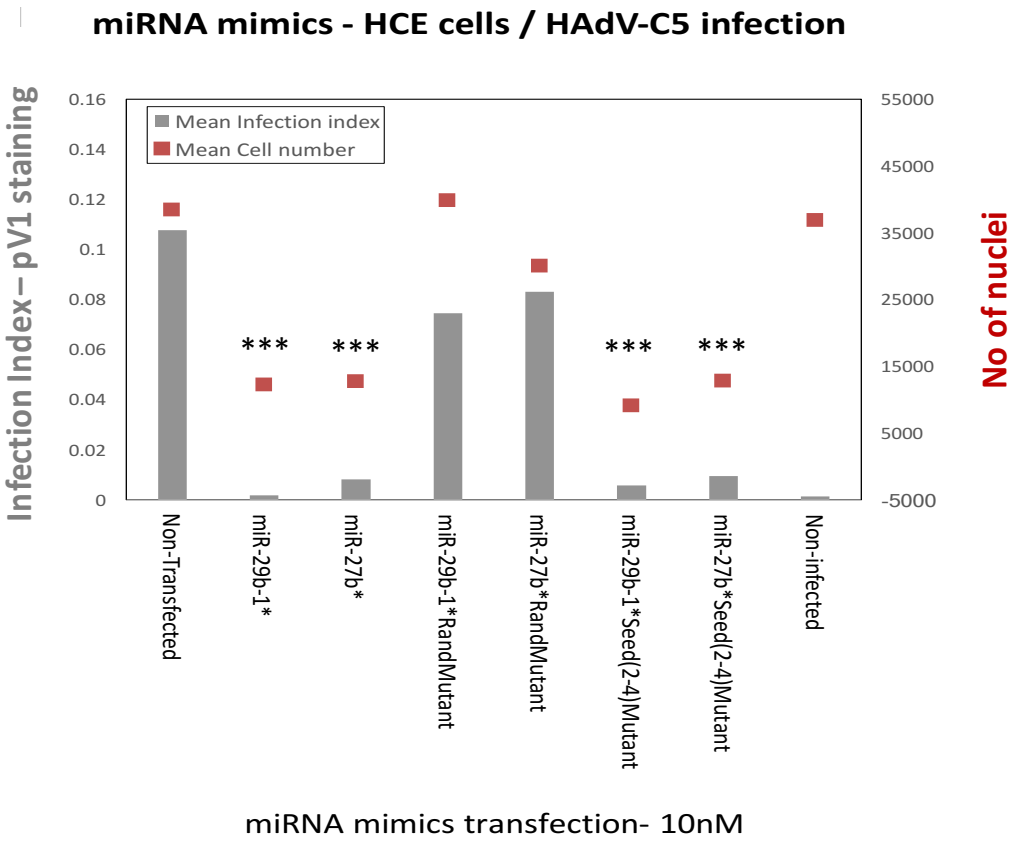
miRNA mimics transfection- 10nM



miRNA mimics transfection- 10nM



miRNA mimics transfection- 0.5M


miRNA mimics - HCE cells / HAdV-C5 infection
Infection Index– pV1 staining
No of nuclei
Mean Infection index
Mean Cell number
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
55000
45000
35000
25000
15000
5000
-5000
***
***
***
***
Non-Transfected
miR-29b-1*
miR-27b*
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*Seed(2-4)Mutant
miR-27b*Seed(2-4)Mutant
Non-infected
miRNA mimics transfection- 10nM

C

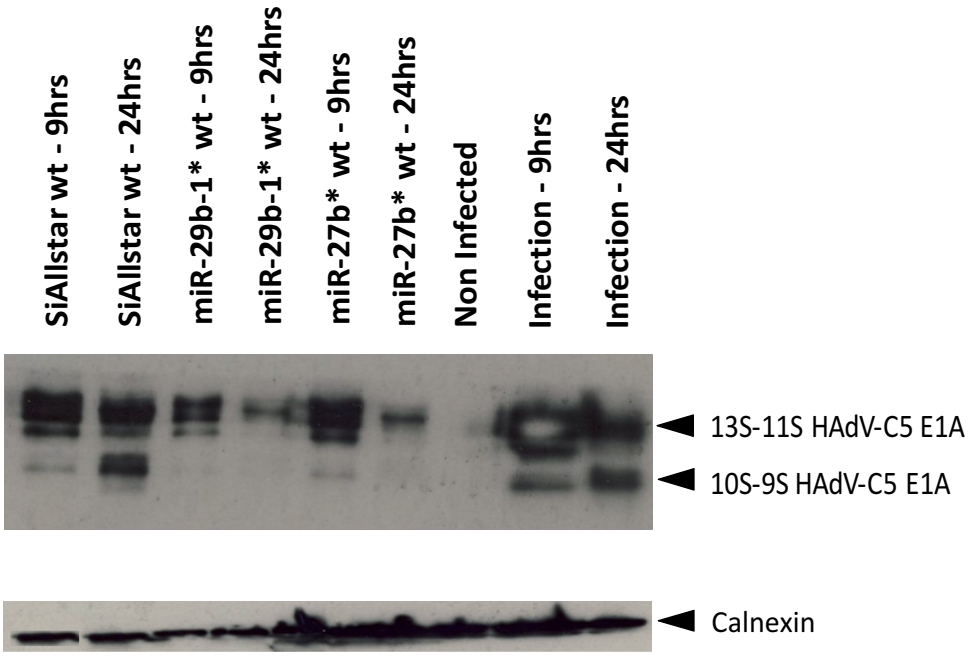
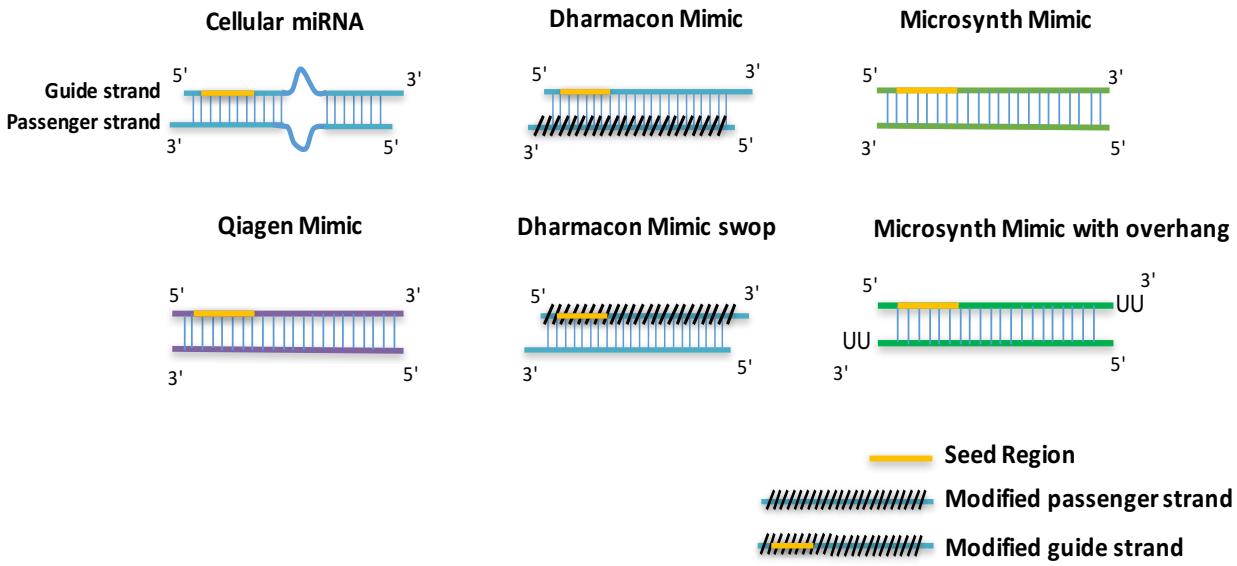
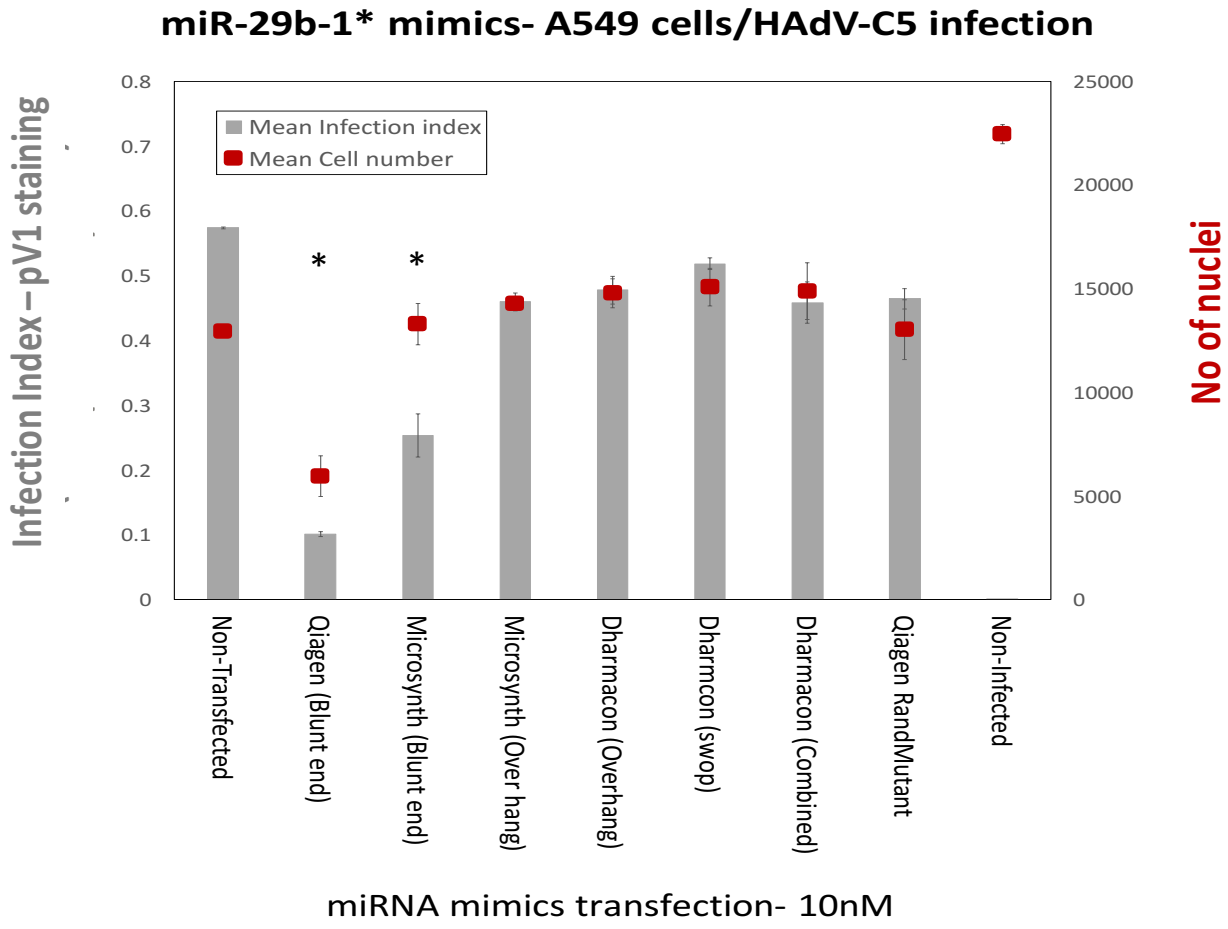

SiAllstar wt - 9hrs
SiAllstar wt - 24hrs
miR-29b-1* wt - 9hrs
miR-29b-1* wt - 24hrs
miR-27b* wt - 9hrs
miR-27b* wt - 24hrs
Non Infected
Infection - 9hrs
Infection - 24hrs
13S-11S HAdV-C5 E1A
10S-9S HAdV-C5 E1A
Calnexin

Figure 2

A


Cellular miRNA
Guide strand
Passenger strand
5'
3'
3'
5'
Dharmacon Mimic
Microsynth Mimic
Qiagen Mimic
Dharmacon Mimic swop
Microsynth Mimic with overhang
UU
UU
Seed Region
Modified passenger strand
Modified guide strand

B


miR-29b-1* mimics- A549 cells/HAdV-C5 infection
Infection Index – pV1 staining
No of nuclei
Mean Infection index
Mean Cell number
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
25000
20000
15000
10000
5000
0
*
*
Non-Transfected
Qiagen (Blunt end)
Microsynth (Blunt end)
Microsynth (Over hang)
Dharmacon (Overhang)
Dharmcon (swop)
Dharmacon (Combined)
Qiagen RandMutant
Non-Infected
miRNA mimics transfection- 10nM

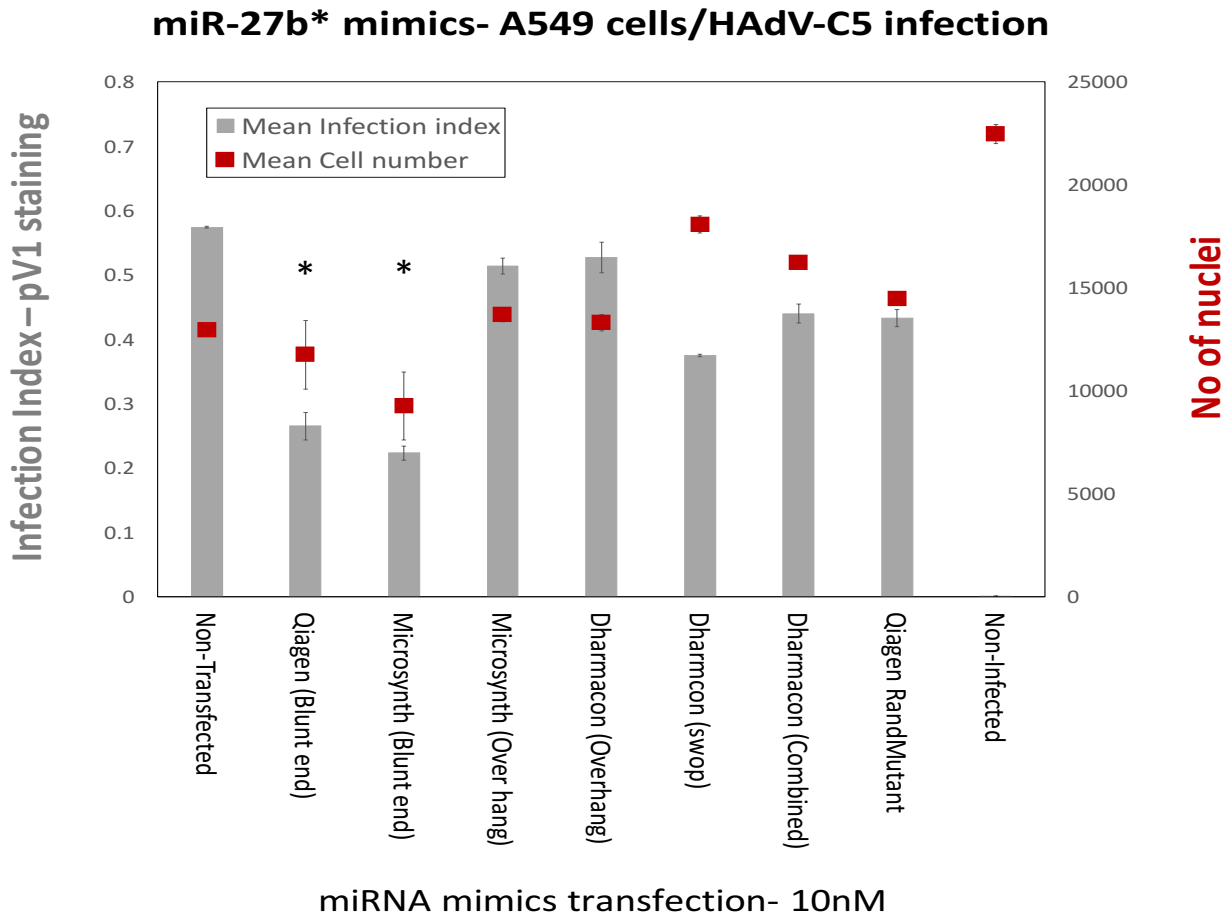
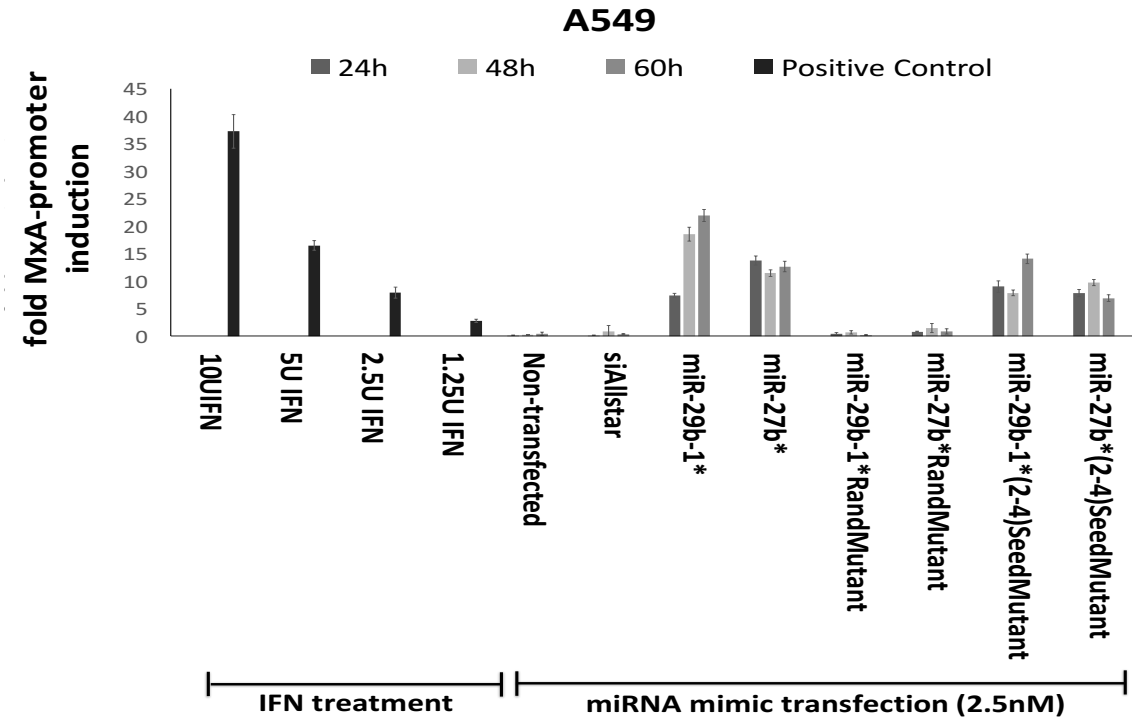

miR-27b* mimics- A549 cells/HAdV-C5 infection
Infection Index – pV1 staining
No of nuclei
Mean Infection index
Mean Cell number
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
25000
20000
15000
10000
5000
0
*
*
Non-Transfected
Qiagen (Blunt end)
Microsynth (Blunt end)
Microsynth (Over hang)
Dharmacon (Overhang)
Dharmcon (swop)
Dharmacon (Combined)
Qiagen RandMutant
Non-Infected
miRNA mimics transfection- 10nM

Figure 3

A


A549
24h
48h
60h
Positive Control
fold MxA-promoter induction
45
40
35
30
25
20
15
10
5
0
10UIFN
5U IFN
2.5U IFN
1.25U IFN
Non-transfected
siAllstar
miR-29b-1*
miR-27b*
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
IFN treatment
miRNA mimic transfection (2.5nM)

B

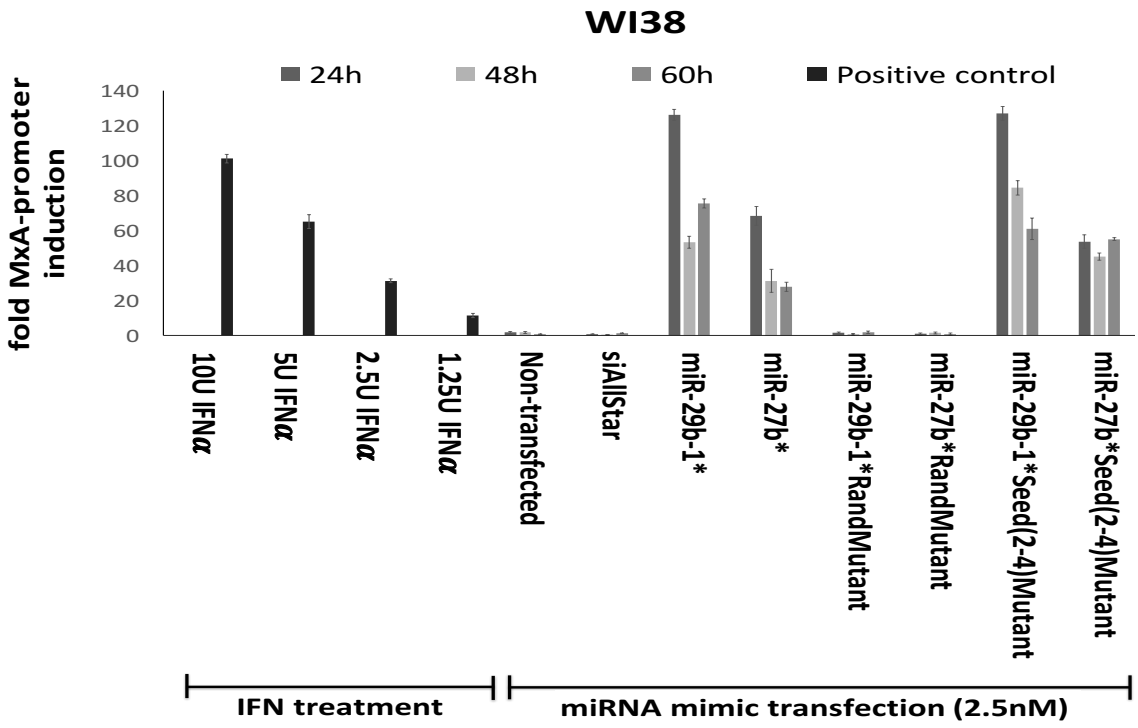
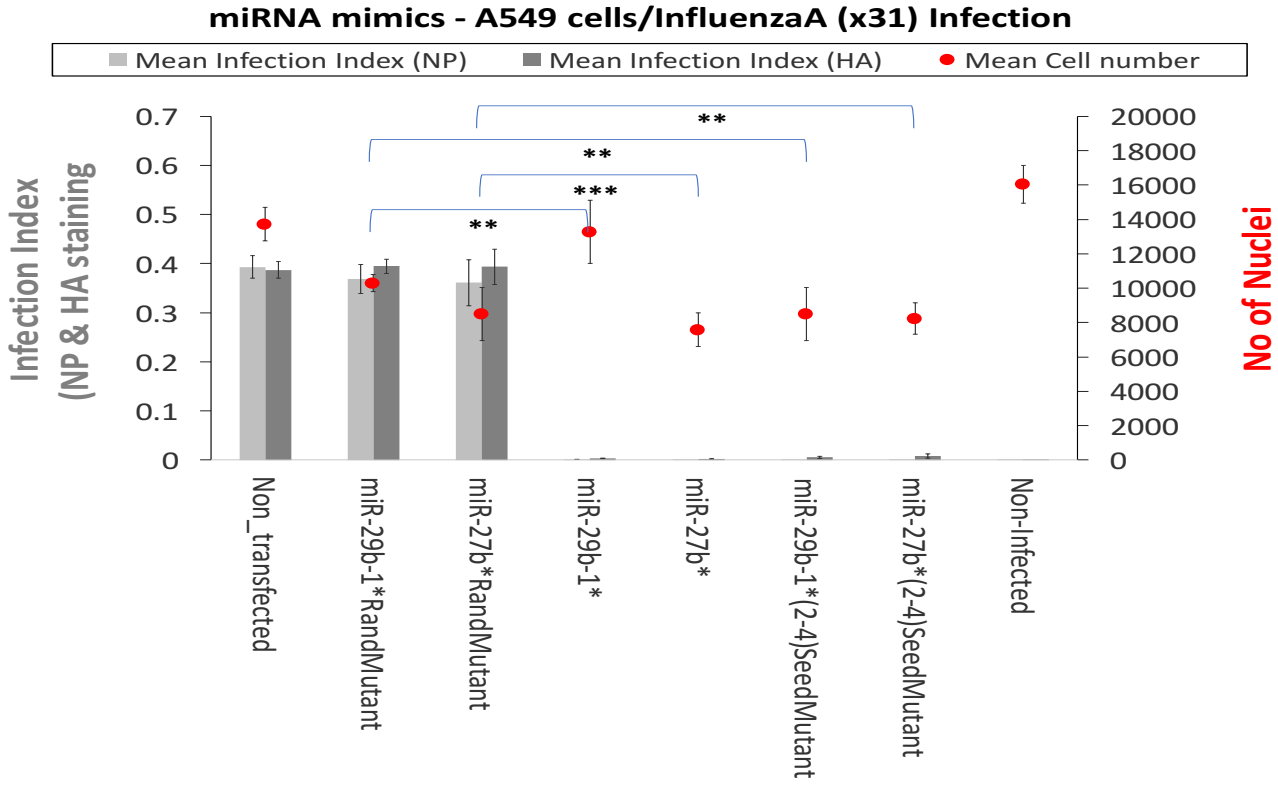
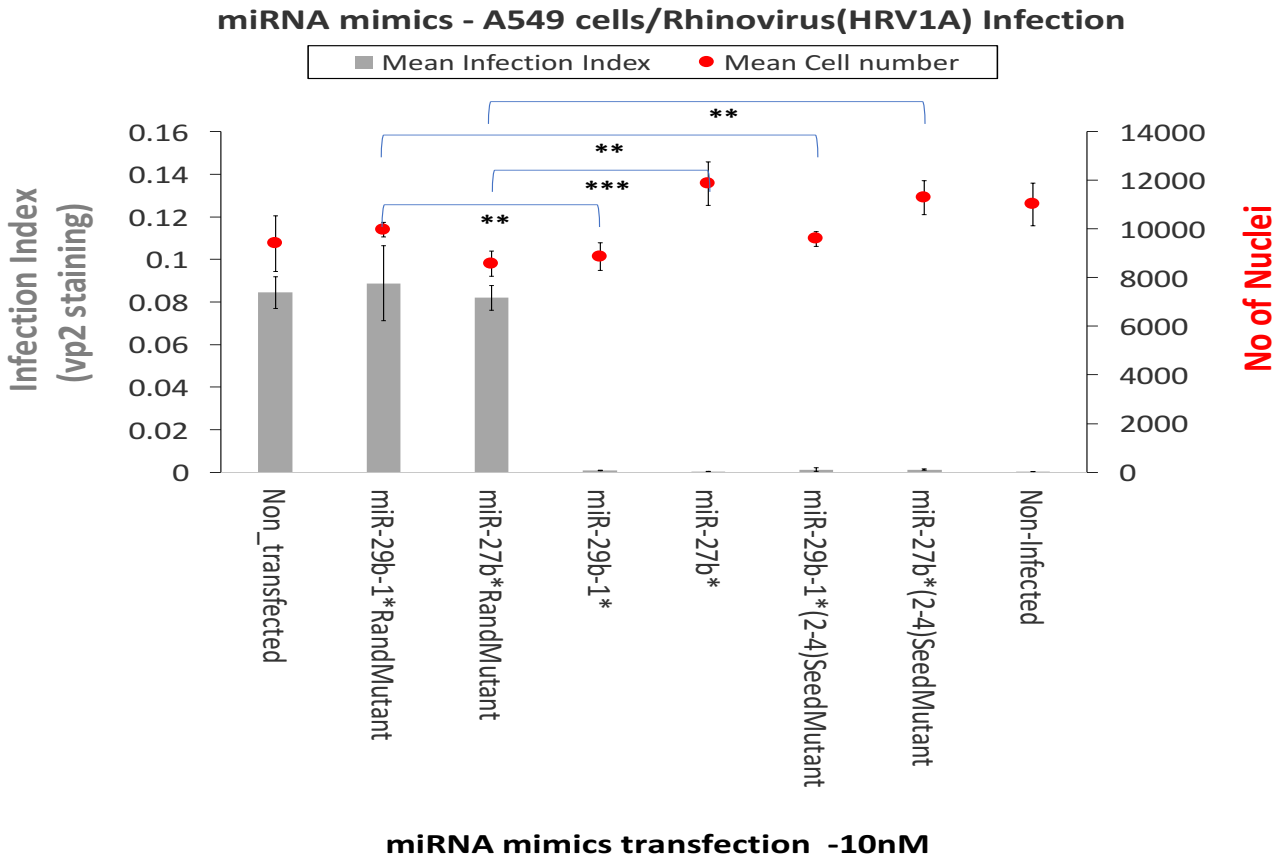

WI38
24h
48h
60h
Positive control
fold MxA-promoter induction
140
120
100
80
60
40
20
0
10U IFNα
5U IFNα
2.5U IFNα
1.25U IFNα
Non-transfected
siAllStar
miR-29b-1*
miR-27b*
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*Seed(2-4)Mutant
miR-27b*Seed(2-4)Mutant
IFN treatment
miRNA mimic transfection (2.5nM)

Figure 4

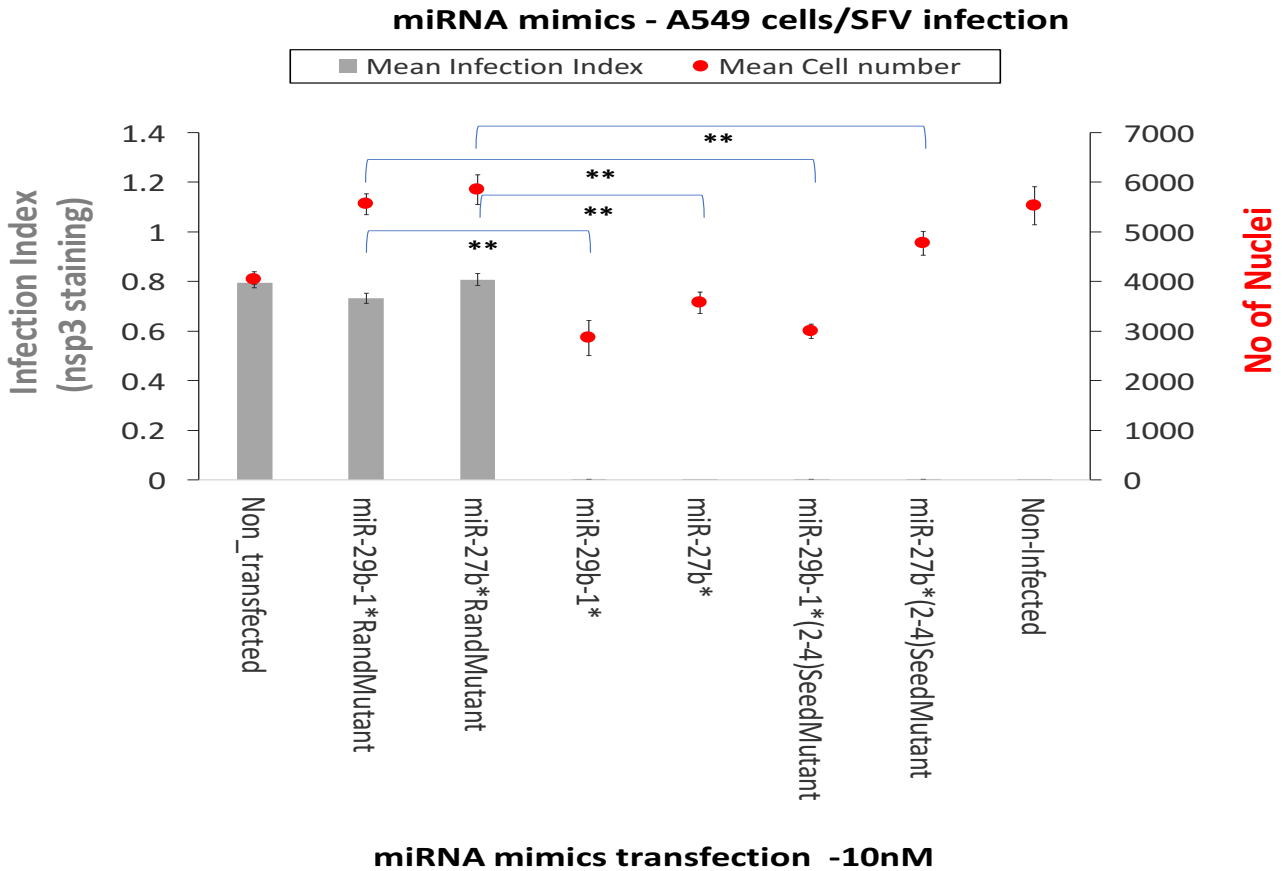
A


miRNA mimics - A549 cells/InfluenzaA (x31) Infection
Mean Infection Index (NP)
Mean Infection Index (HA)
Mean Cell number
Infection Index (NP & HA staining
No of Nuclei
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
**
**
***
**
Non_transfected
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
Non-Infected
miRNA mimics transfection -10nM

B


miRNA mimics - A549 cells/Rhinovirus(HRV1A) Infection
Mean Infection Index
Mean Cell number
Infection Index (vp2 staining)
No of Nuclei
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
14000
12000
10000
8000
6000
4000
2000
0
**
**
***
**
Non_transfected
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
Non-Infected
miRNA mimics transfection -10nM

C


miRNA mimics - A549 cells/SFV infection
Mean Infection Index
Mean Cell number
Infection Index (nsp3 staining)
No of Nuclei
1.4
1.2
1
0.8
0.6
0.4
0.2
0
7000
6000
5000
4000
3000
2000
1000
0
**
**
**
**
Non_transfected
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
Non-Infected
miRNA mimics transfection -10nM

D

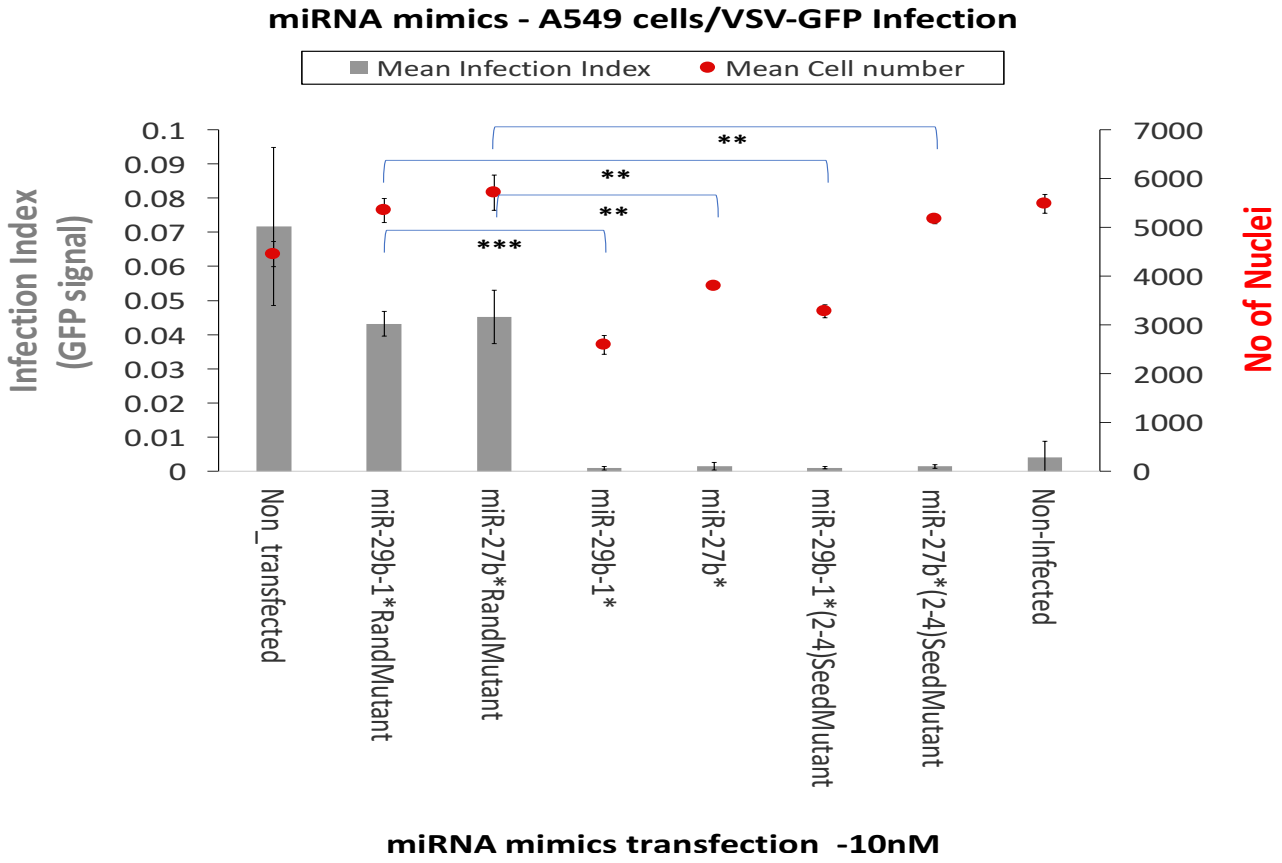
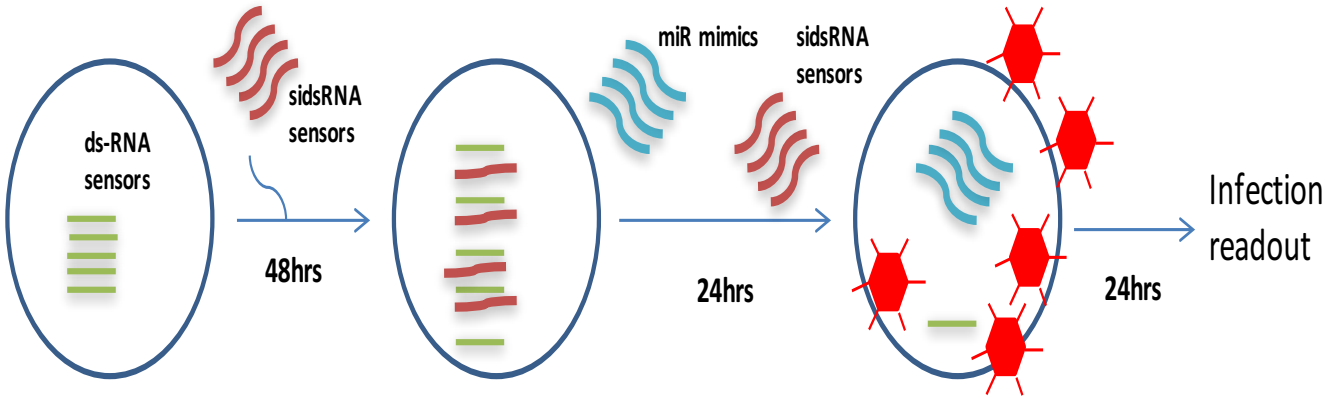
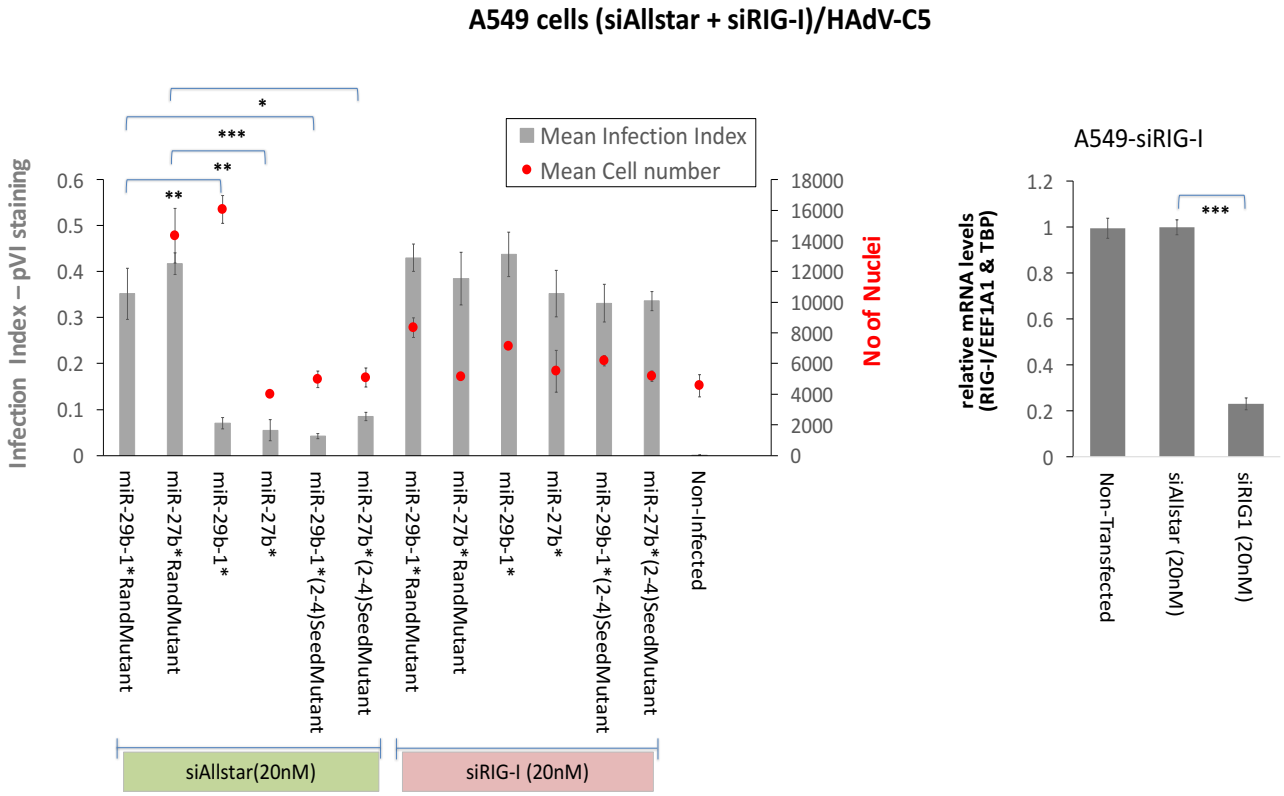

miRNA mimics - A549 cells/VSV-GFP Infection
Mean Infection Index
Mean Cell number
Infection Index (GFP signal)
No of Nuclei
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
7000
6000
5000
4000
3000
2000
1000
0
**
**
**
***
Non_transfected
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
Non-Infected
miRNA mimics transfection -10nM

Figure 5

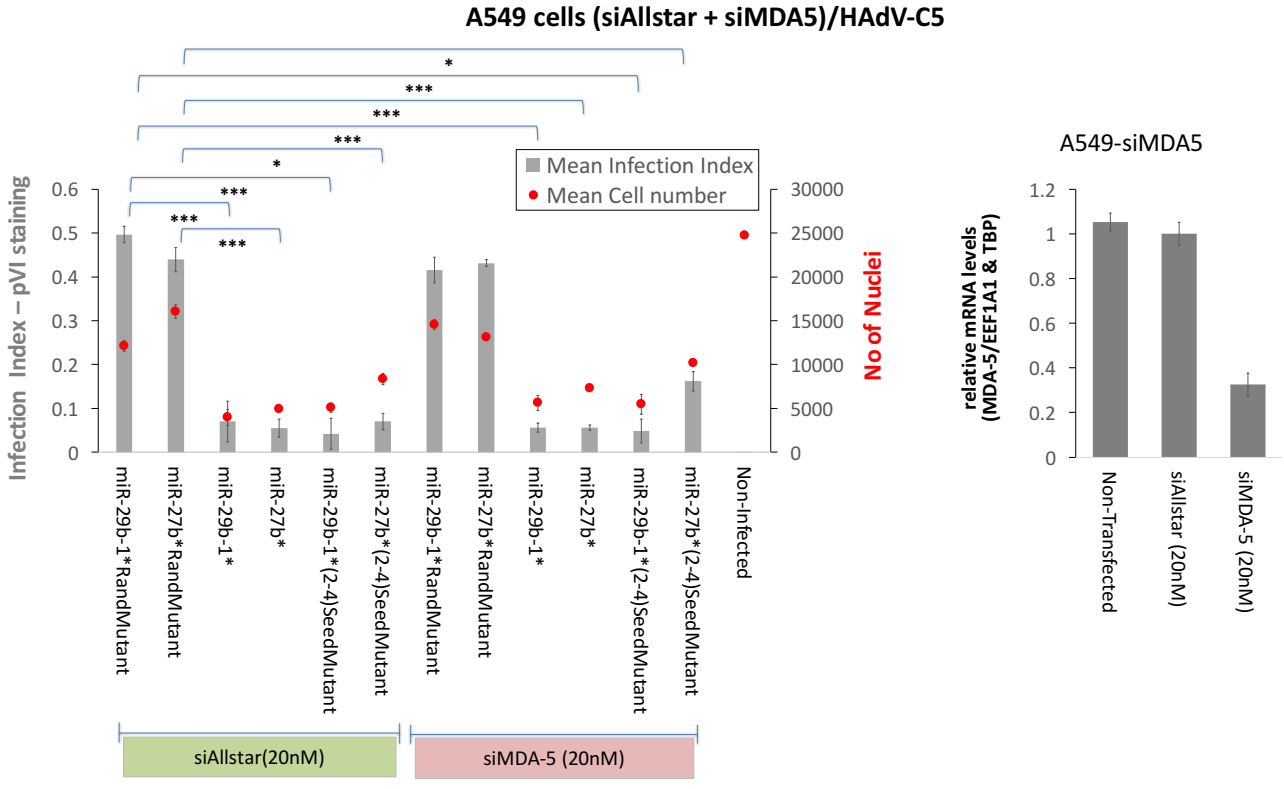
A


ds-RNA sensors
sidsRNA sensors
48hrs
miR mimics
sidsRNA sensors
24hrs
24hrs
Infection readout

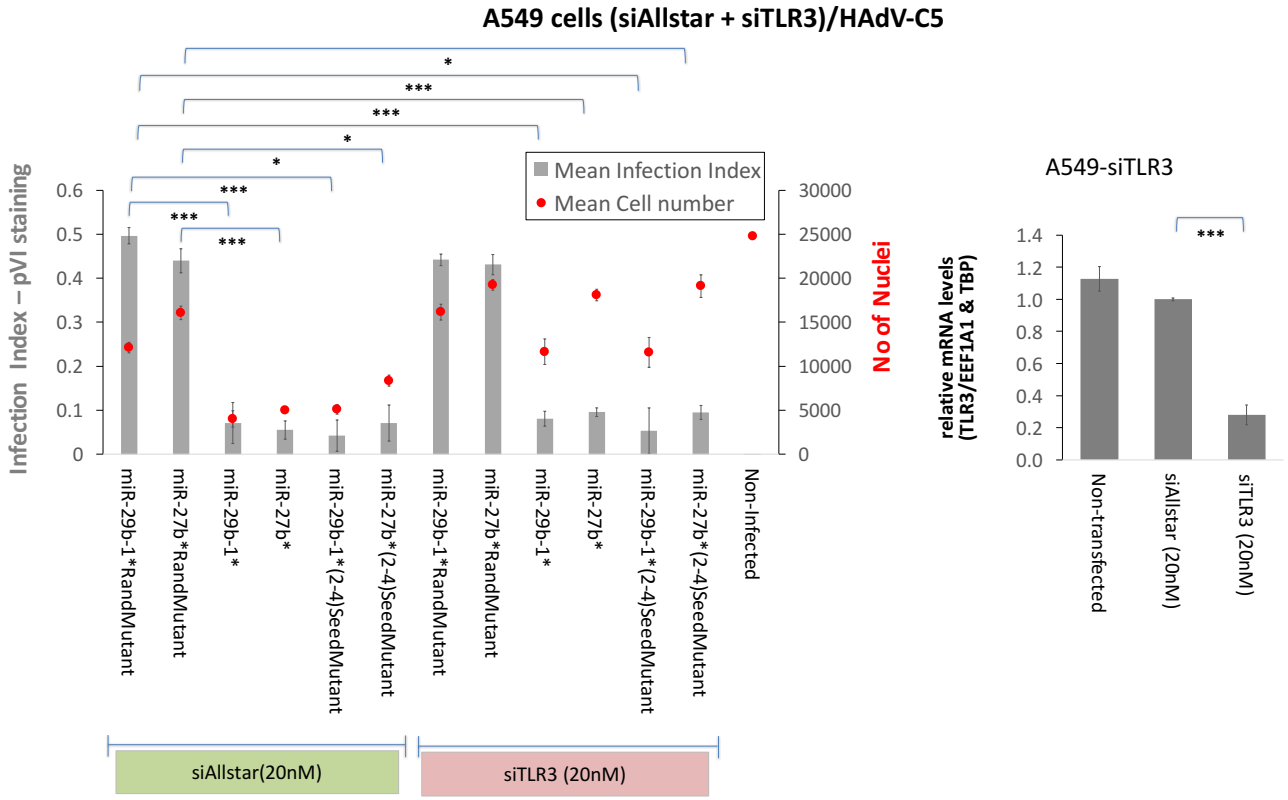
B


A549 cells (siAllstar + siRIG-I)/HAdV-C5
Mean Infection Index
Mean Cell number
Infection Index – pVI staining
No of Nuclei
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
Non-Infected
siAllstar(20nM)
siRIG-I (20nM)
A549-siRIG-I
relative mRNA levels (RIG-I/EEF1A1 & TBP)
Non-Transfected
siAllstar (20nM)
siRIG1 (20nM)

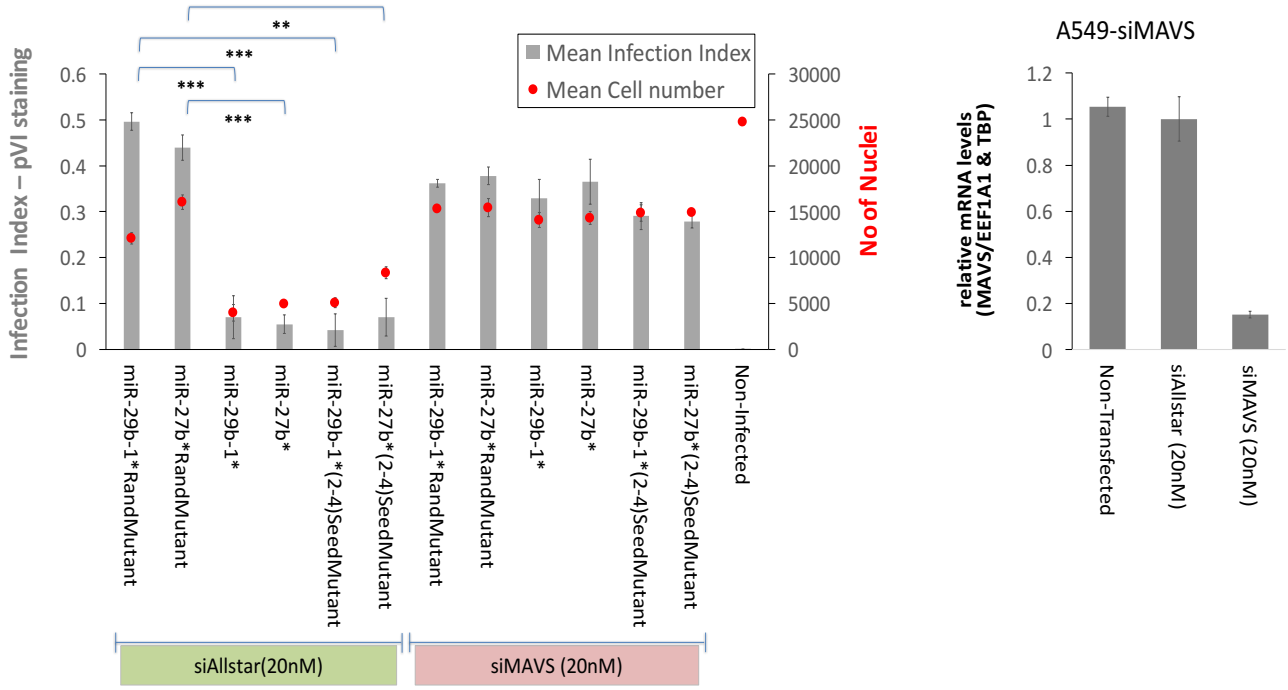
C


A549 cells (siAllstar + siMDA5)/HAdV-C5
Mean Infection Index
Mean Cell number
Infection Index – pVI staining
No of Nuclei
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
Non-Infected
siAllstar(20nM)
siMDA-5 (20nM)
A549-siMDA5
relative mRNA levels (MDA-5/EEF1A1 & TBP)
Non-Transfected
siAllstar (20nM)
siMDA-5 (20nM)

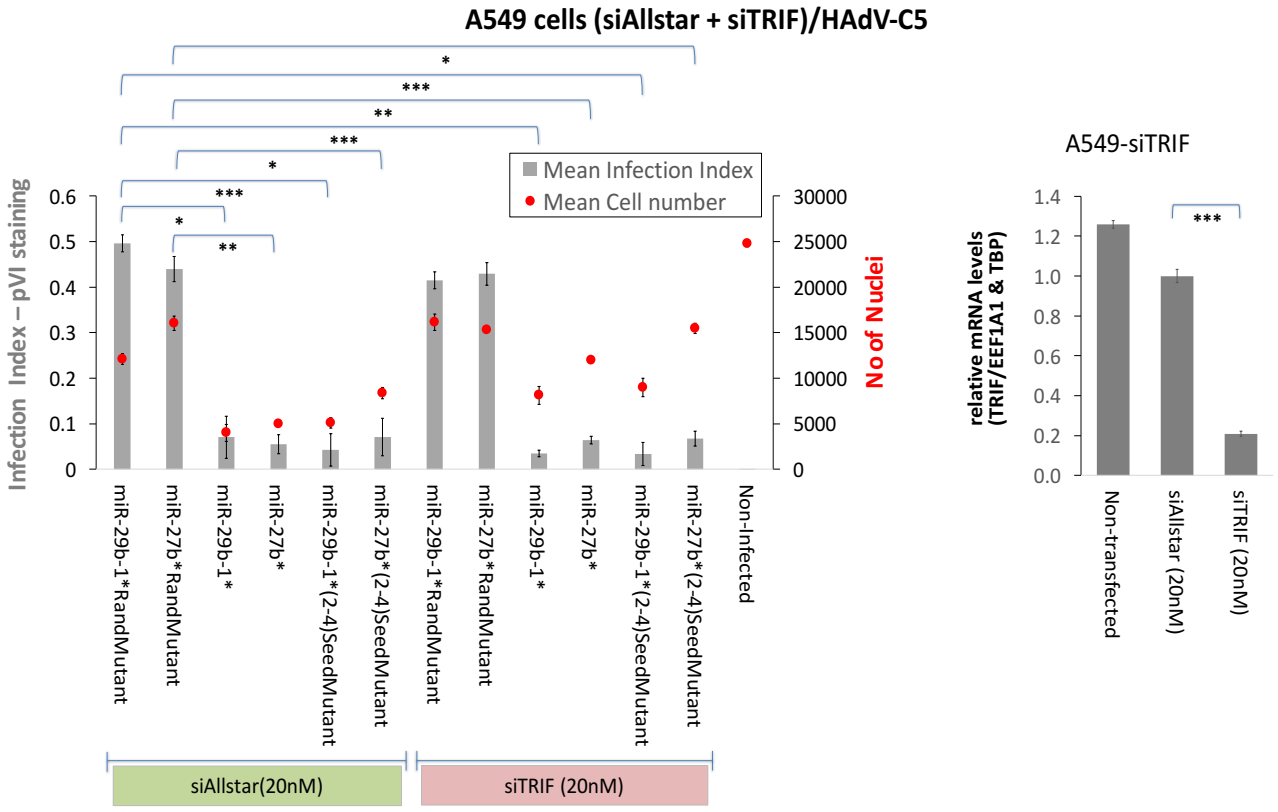
D


A549 cells (siAllstar + siTLR3)/HAdV-C5
Infection Index – pVI staining
No of Nuclei
Mean Infection Index
Mean Cell number
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
Non-Infected
siAllstar(20nM)
siTLR3 (20nM)
A549-siTLR3
relative mRNA levels (TLR3/EEF1A1 & TBP)
Non-transfected
siAllstar (20nM)
siTLR3 (20nM)

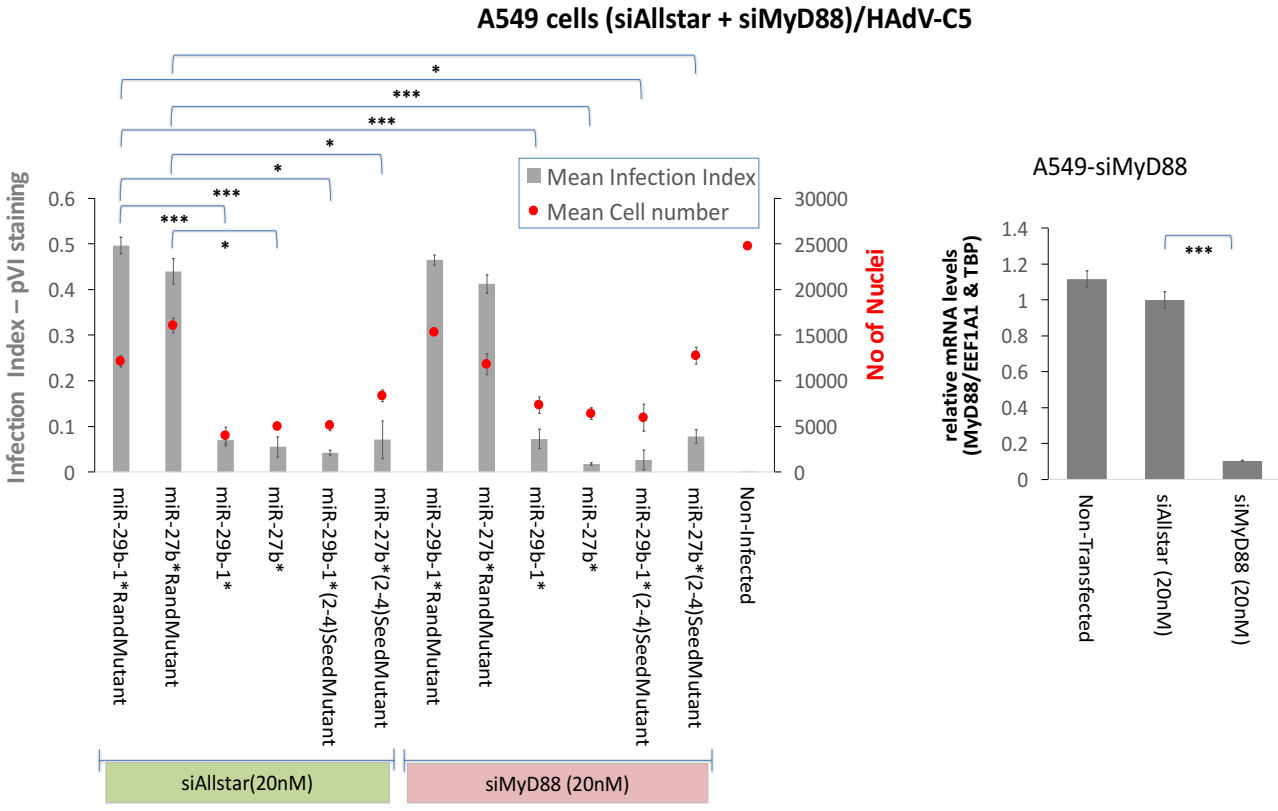
E


A549 cells (siAllstar + siMAVs)/HAdV-C5
Infection Index – pVI staining
No of Nuclei
Mean Infection Index
Mean Cell number
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
Non-Infected
siAllstar(20nM)
siMAVS (20nM)
A549-siMAVS
relative mRNA levels (MAVS/EEF1A1 & TBP)
Non-Transfected
siAllstar (20nM)
siMAVS (20nM)

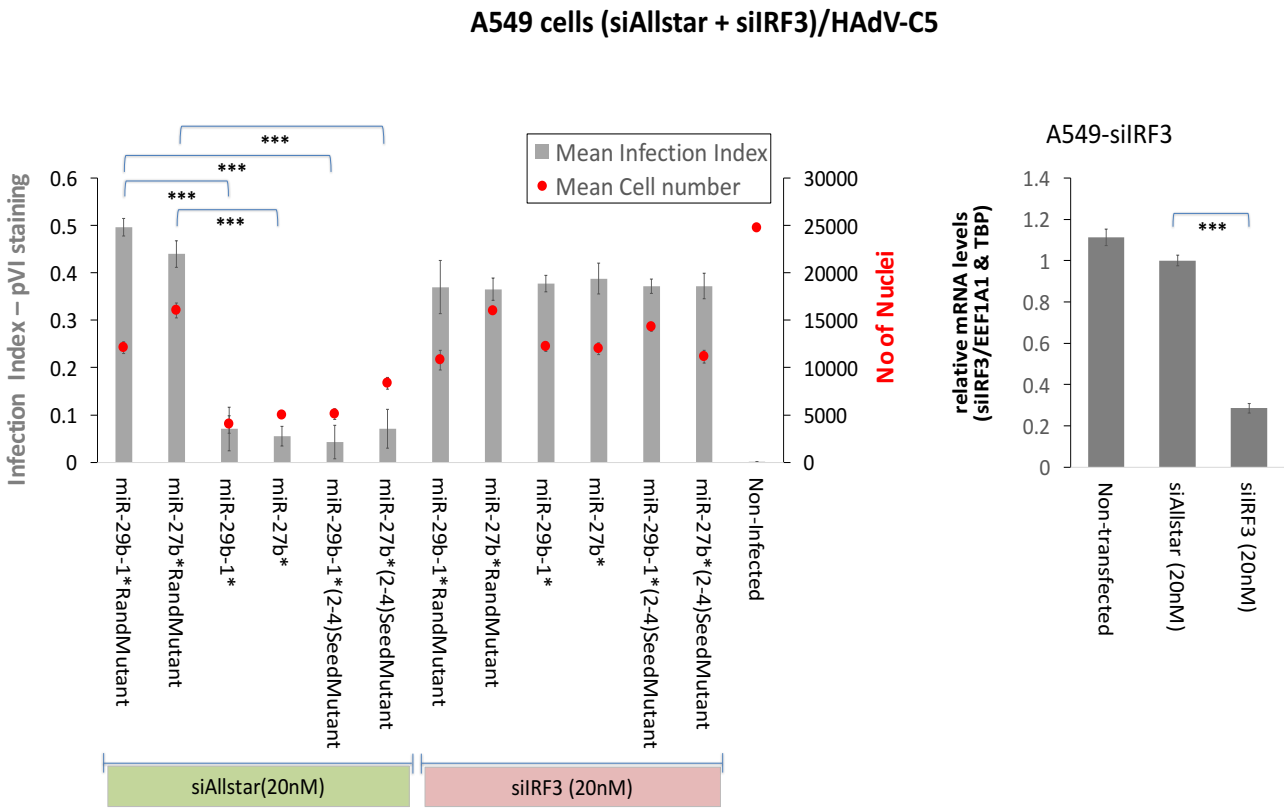
F


A549 cells (siAllstar + siTRIF)/HAdV-C5
Mean Infection Index
Mean Cell number
Infection Index – pVI staining
No of Nuclei
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
Non-Infected
siAllstar(20nM)
siTRIF (20nM)
A549-siTRIF
relative mRNA levels (TRIF/EEF1A1 & TBP)
Non-transfected
siAllstar (20nM)
siTRIF (20nM)

G


A549 cells (siAllstar + siMyD88)/HAdV-C5
Mean Infection Index
Mean Cell number
Infection Index – pVI staining
No of Nuclei
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
Non-Infected
siAllstar(20nM)
siMyD88 (20nM)
A549-siMyD88
relative mRNA levels (MyD88/EEF1A1 & TBP)
Non-Transfected
siAllstar (20nM)
siMyD88 (20nM)

H


A549 cells (siAllstar + siIRF3)/HAdV-C5
Mean Infection Index
Mean Cell number
Infection Index – pVI staining
No of Nuclei
***
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
Non-Infected
siAllstar(20nM)
siIRF3 (20nM)
A549-siIRF3
relative mRNA levels (siIRF3/EEF1A1 & TBP)
Non-transfected
siAllstar (20nM)
siIRF3 (20nM)

I

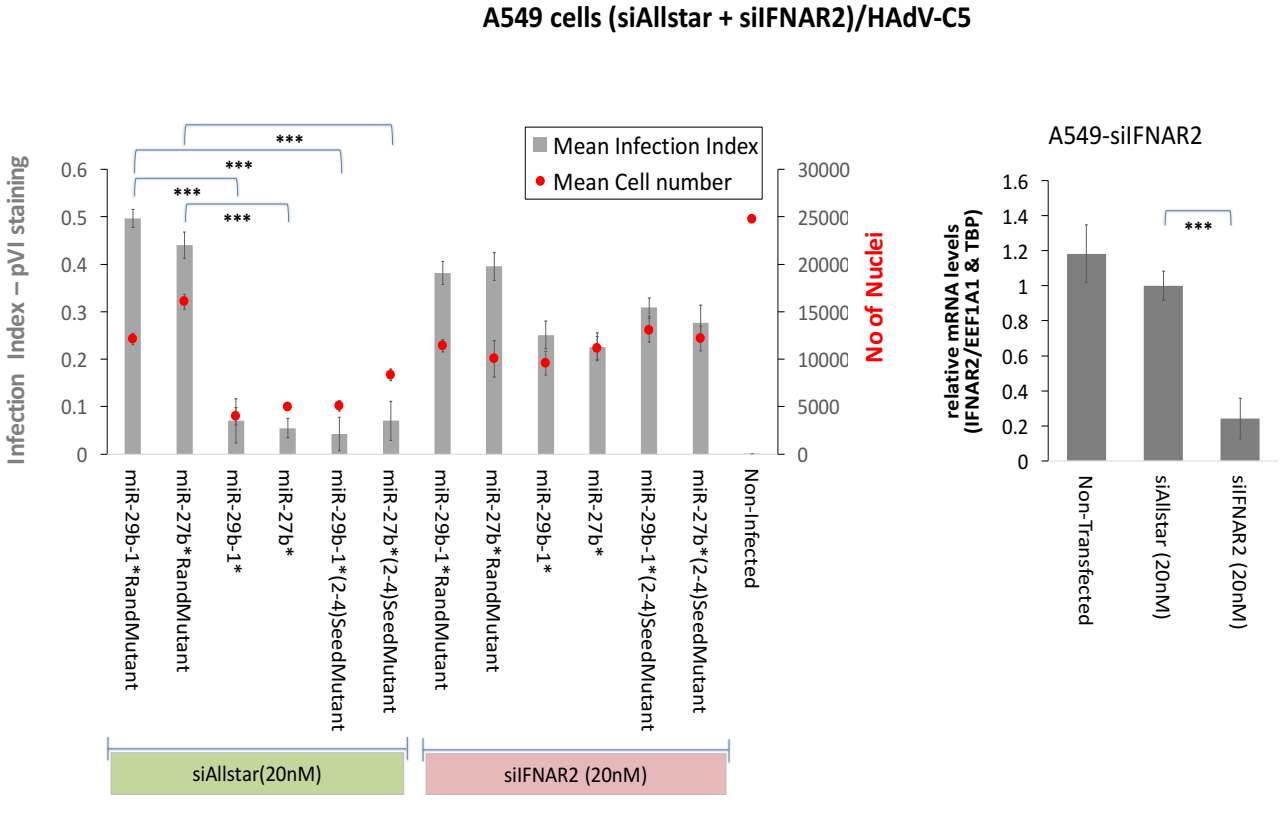
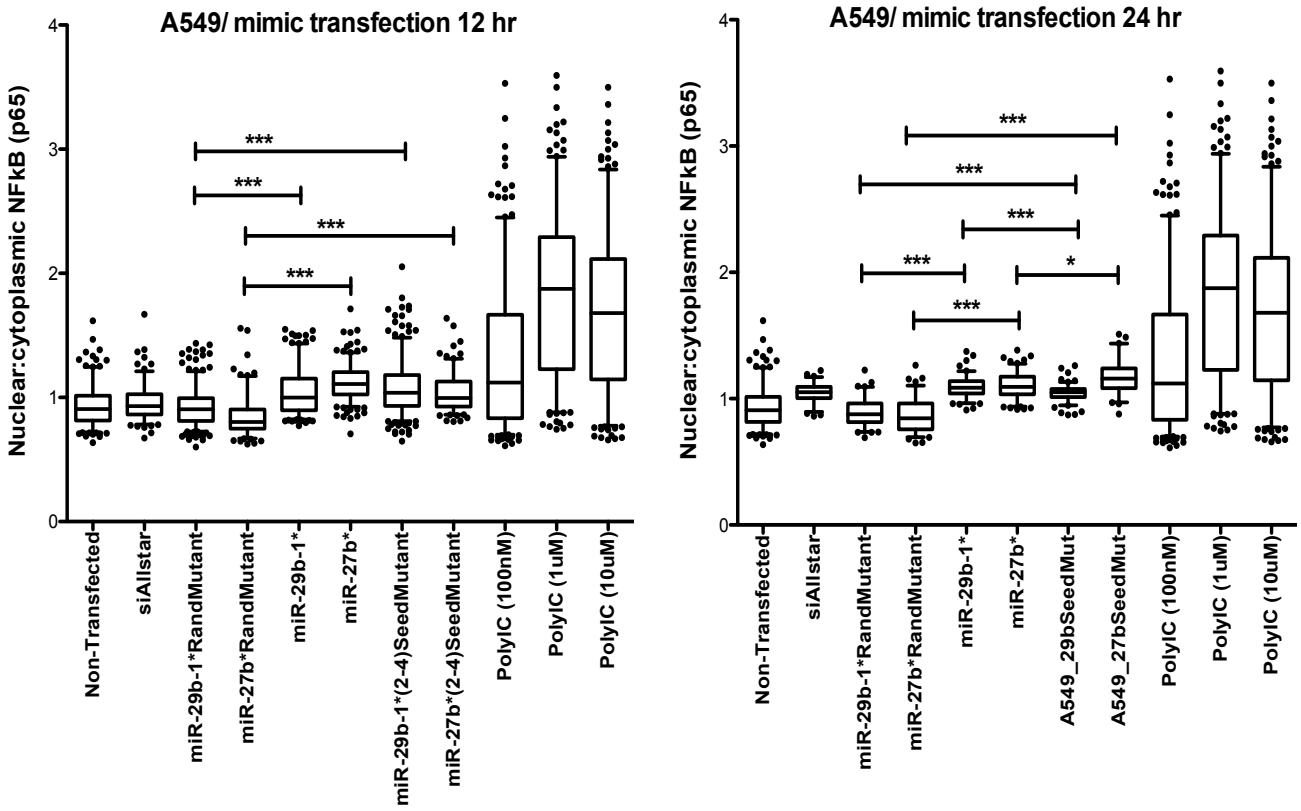

A549 cells (siAllstar + siIFNAR2)/HAdV-C5
Mean Infection Index
Mean Cell number
Infection Index – pVI staining
No of Nuclei
***
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
Non-Infected
siAllstar(20nM)
siIFNAR2 (20nM)
A549-siIFNAR2
relative mRNA levels (IFNAR2/EEF1A1 & TBP)
Non-Transfected
siAllstar (20nM)
siIFNAR2 (20nM)

Figure 6

A


A549/ mimic transfection 12 hr
Nuclear:cytoplasmic NFkB (p65)
Non-Transfected
siAllstar
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
PolyIC (100nM)
PolyIC (1uM)
PolyIC (10uM)
A549/ mimic transfection 24 hr
Nuclear:cytoplasmic NFkB (p65)
Non-Transfected
siAllstar
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
A549_29bSeedMut
A549_27bSeedMut
PolyIC (100nM)
PolyIC (1uM)
PolyIC (10uM)

B

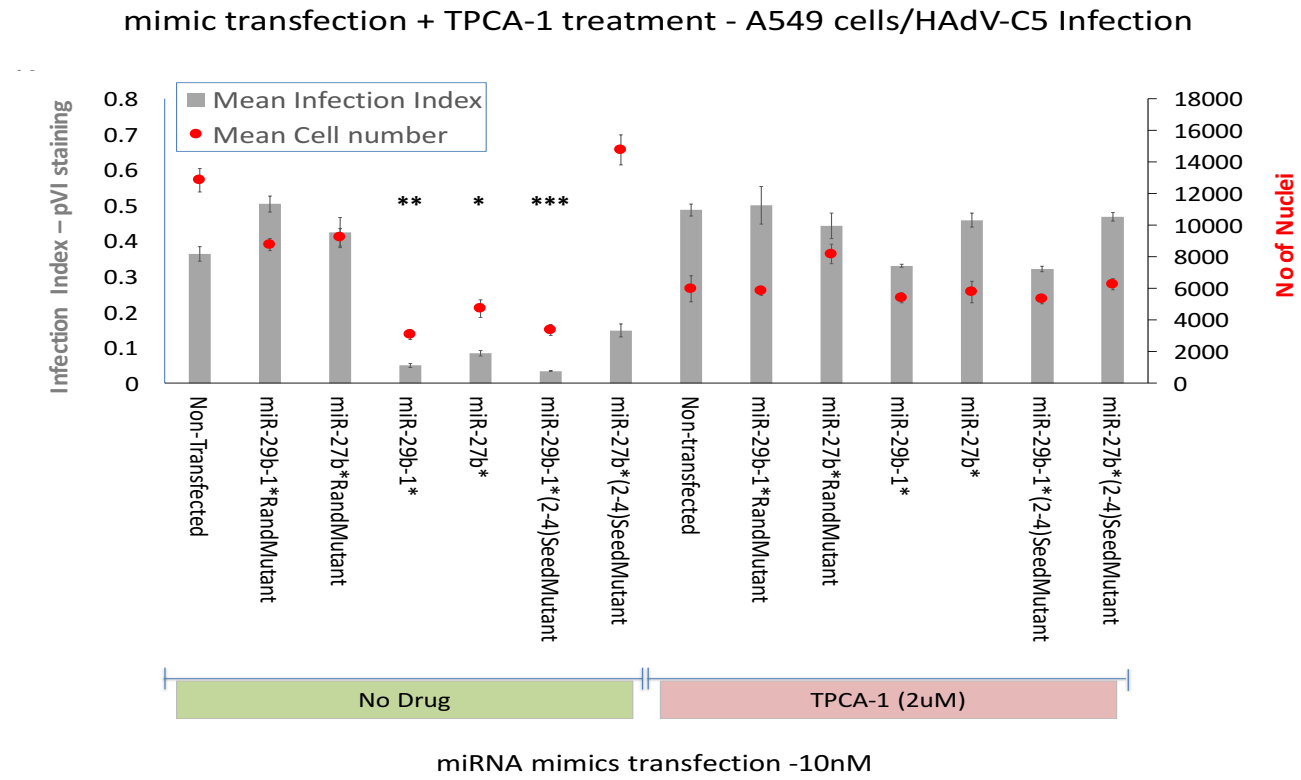
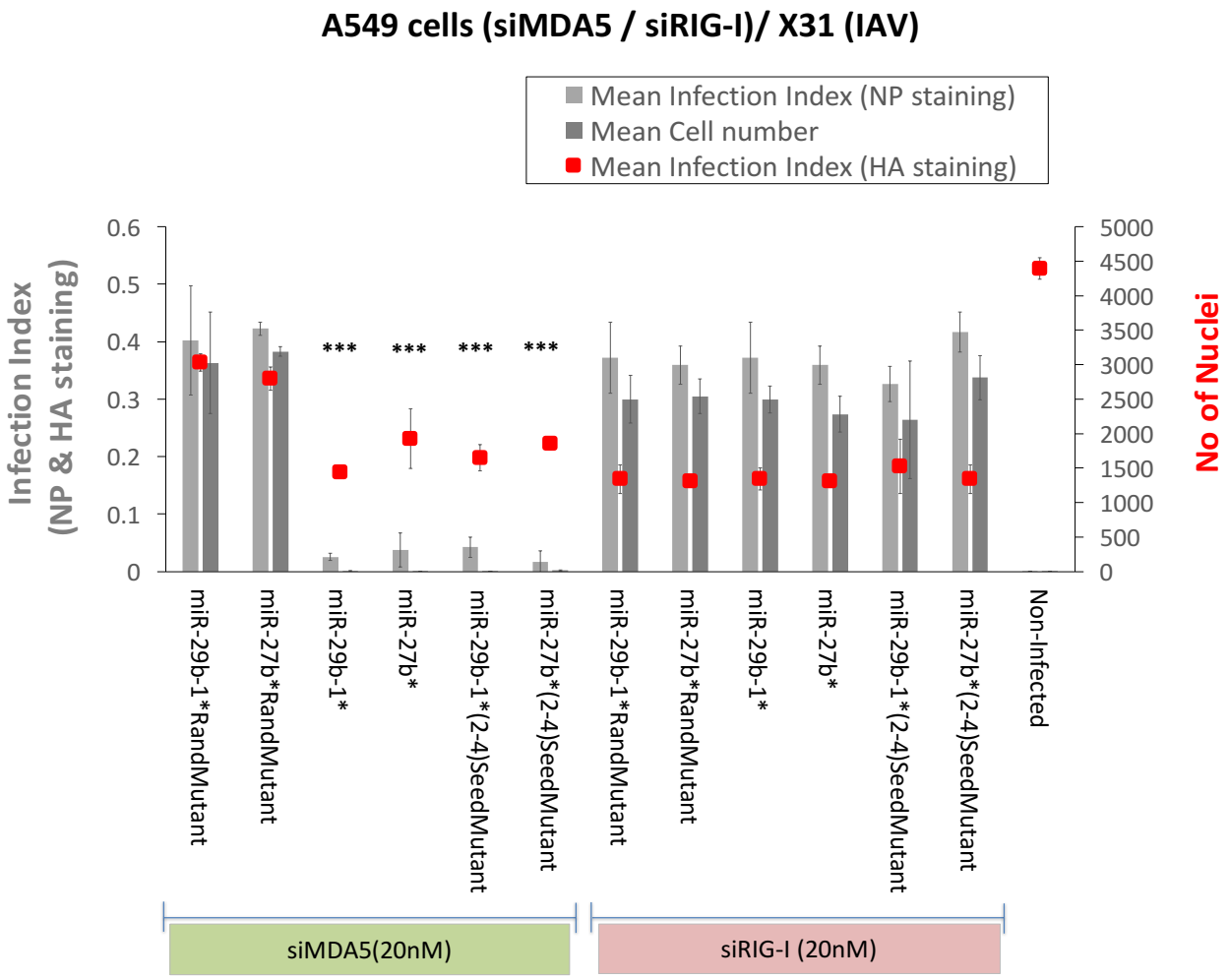

mimic transfection + TPCA-1 treatment - A549 cells/HAdV-C5 Infection
Infection Index – pVI staining
Mean Infection Index
Mean Cell number
No of Nuclei
Non-Transfected
miR-29b-1*RandMutant
miR-27b*RandMutant
miR-29b-1*
miR-27b*
miR-29b-1*(2-4)SeedMutant
miR-27b*(2-4)SeedMutant
No Drug
TPCA-1 (2uM)
miRNA mimics transfection -10nM

Table S1

miRNA mimic	Sequence 5' -> 3' / comments	ID / Cat. Nr.
Syn-hsa-miR-27b* miScript miRNA Mimic	AGAGCUUAGCUGAUUGGUGAAC	MSY0004588
Syn-hsa-miR-29b-1* miScript miRNA Mimic	GCUGGUUUCAUAUGGUGGUUUAGA	MSY0004514
Syn-hsa-miR-29b-1*Seed Mutant	GGCUGUUUCAUAUGGUGGUUUAGA	Custom made
Syn-hsa-miR-27b*Seed Mutant	AUUUCUUAGCUGAUUGGUGAAC	Custom made
Syn-hsa-miR-29b-1*Randomized Mutant	UUUAUGCGGGGUGUCGUAUAAUGU	Custom made
Syn-hsa-miR-27b*Randomized Mutant	UAGAUGAGAGAUUCGGCUGUCA	Custom made

Figure S1



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Chapter 4

Conclusions and outlook

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miRNAs are key regulators of host defence response against viruses (Chang et al. 2011; Querec et al. 2009; Diercks & Aderem 2012). Expression of miRNAs in the host cells are regulated by many viruses which help them to promote key pro-viral processes such as prolonged survival of infected cells, pathogenesis and replication (Guo & Steitz 2014). However, pathogen driven miRNA overexpression could sometimes be specific to a particular cell or tissue and may not necessarily correlate with viral pathogenesis and survival of the infected cell. The precise mechanism by which viruses regulate their miRNAs, specific virulence factors and host miRNAs is unclear to a large extent and needs more validations. Novel approaches to develop antiviral therapeutics requires detailed understanding of molecular mechanism(s) of viral infection, virus mediated regulation of host miRNAs and vice versa. miRNA inhibitors or miRNA analogues such as miRNA mimics which modulate miRNA expression can be utilized in the development of prophylactic vaccines or new therapies for viral infections. Additionally, miRNAs could be used as biomarkers for prognosis and laboratory diagnosis (Fredsøe et al. 2017; Pal et al. 2015). Thorough understanding of host miRNA specific functions in pathogen host interactions is a key step in identifying miRNA targets. Overall, miRNAs can serve as important tools for therapeutic and diagnostic innovations against infections. The initial goal of the PhD project was to characterize host miRNAs which were differentially regulated by human adenoviruses and to study their impact on cellular factors. With this regard, several host miRNAs were found to be differentially regulated upon HAdV infections (explained in **chapter 2** of the thesis). However, our further studies were mainly focussed on miR-29b-1* and miR-27b*, as these two miRNAs were confirmed to be strongly downregulated by both HAdV-C5 and HAdV-B3.

In order to predict the possible viral targets of these miRNAs, an in-silico approach was sought, where sequence complementarity was assessed between the miRNA and viral genes. Based on this approach, several key HAdV genes, including E1B, pIX, E3, Iva2 and pol were predicted to be the potential targets of miR-29b-1* and miR-27b*. Additionally some of the host factors (involved in HAdV-C5 entry) like CXADR and ITGAV were assumed to be the predicted targets of these miRNAs based on in-silico binding energies obtained from miRNA-target interactions as described in (Khorshid et al. 2013). Both canonical miRNA seed based and the secondary non-canonical whole miRNA sequence based interactions with the target were considered for the predictions. To understand the functional relevance of miR-29b-1* and miR-27b* in HAdV infection, transcriptome analysis of A549 cells was performed with commercially synthesized mimics of miR-29b-1* and miR-27b* to identify host genes differentially regulated by these miRNAs. These mimics induced a vigorous type I interferon response, as indicated by the upregulation of multiple innate immune response and interferon–induced genes. Additionally, Cell adhesion and cell cycle genes were strongly downregulated by these mimics. The mimics used in this study were initially obtained from Qiagen and were double stranded,

non-modified blunt end molecules. These mimics, upon transfection significantly inhibited HAdV infection of cultured cells. Interestingly, mimics with the mutated seed sequences also inhibited HAdV-C5 infection significantly. These findings led to following possibilities: 1) miRNA mimics could truly imitate endogenous mature miRNA duplexes, 2) It could be advantageous for the virus to downregulate miR-29b-1* and miR-27b* due to the anti-adenoviral activity of these mimics, 3) Since the induction of type 1 IFN response was observed with these mimics, the downregulation of miR-29b-1* and miR-27b* could possibly suppress IFN/cytokines production and this could promote virus spreading without being recognized by host immune system, 4) overall sequence of the mimic was important for the effect.

Major question however, was to determine if these mimics truly acted as functional equivalents to the endogenous miRNAs. In order to claim that miR-29b-1* and miR-27b* inhibits HAdV infection, it was necessary to observe anti-adenoviral effects with all the different types of miR29b-1* and miR-27b* mimics obtained from different commercial vendors. However, only blunt ended, non-modified mimics of miR-29b-1* and miR-27b* obtained from Qiagen and Microsynth were successful in inhibiting HAdV infection (explained in **chapter 3** of the thesis). These findings indicated that the mimics did not function as endogenous host miR-29b-1* and miR-27b*. Additionally, these mimics consist of two completely complementary strands, whereas an endogenous miRNA consists of a guide and passenger strand and lack complete complementarity between each other. dsRNA molecules have been extensively studied over the past decade primarily as an inducer of innate immune response and important modulator of viral pathogenesis (Karpala et al. 2005; Nellimarla & Mossman 2014; Gantier & Williams 2007; Dewitte-Orr & Mossman 2010). Immune response triggered by dsRNA serves to control viral infection in the host. These properties make short dsRNA mimics a good candidate for antiviral therapeutics. With the discovery of poly(I:C) (Polyinosinic: polycytidylic acid), a mismatched dsRNA molecule in 1960s, it was considered to be the 'wonder drug' for treating virus infection (Kato et al. 2008; Palchetti et al. 2015; Fortier et al. 2004). Poly (I:C) based drugs, namely Ampligen and Hiltonol were used in the clinical trials (Fortier et al. 2004; Brodsky et al. 1985; Champney et al. 1979). Even after showing promising effects, these drugs were not approved by FDA due to high toxicity associated with these molecules. Host responses induced by these drugs did not always correlate to the effects shown by viral dsRNA. miR-29b-1* and miR-27b* mimics used in this work, upon transfection resulted in the production of type I IFNs by the cultured cells. In addition to HAdV-C5, these blunt ended, non-modified qiagen mimics of miR-29b-1* and miR-27b* significantly suppressed several viral infections including IAV, HRV1A, SFV and VSV. This implied that that these short dsRNA mimics could induce innate immune sensing in the cells and thereby exhibit broad spectrum antiviral activity. With this regard, siRNA mediated knockdown of several key dsRNA sensors and their effector molecules were carried out. The results clearly suggested that these mimics either directly or indirectly activated RIG-I and this was probably the reason for the broad spectrum antiviral response shown by the mimics. Additionally, it was interesting to note the presence of specific 'UGGU' motif in

these mimics. Since only these mimics, and not the controls (For example, Randomized mutants and the Qiagen mimics of other HAdV downregulated miRNAs), led to the strong inhibition of viral infection, the antiviral activity of these mimics could possibly be attributed to the sequence-specific activation of RIG-I-dependent innate immune response. However, this requires careful mapping and characterization of the activating sequence in the non-seed region of the mimic. Based on the above findings, such short blunt ended, non-modified mimics with specific sequential features could serve as a good candidate for antiviral therapeutics. However, it is important to determine how these mimics are able to activate RIG-I (i.e. if these mimics can directly be recognized by RIG-I). This was addressed by assaying ATPase activity of RIG-I in-vitro in the presence of miR-29b-1* and miR-27b* mimics. However, the results obtained from this assay was inconclusive. An alternative approach to the in-vitro assay would be to express tagged RIG-I in cells, perform affinity-purification of RIG-I and determine co-purified RNA molecules by next generation sequencing (as described by Sanchez David et al. 2016). Instead of RIG-I, these blunt ended, non-modified dsRNA mimics could also be recognized by other dsRNA sensors operating upstream of RIG-I. There also exists a possibility that these mimics neutralize the negative regulator of RIG-I by binding to it. These questions could be addressed by carrying out siRNA – mediated knockdown of RIG-I interaction partners including LGP2, DDX3, DHX9, DDX60, OAS-proteins/RNAseL, PACT and ZAPS (Fullam & Schröder 2013) and thereby assess its effect on the mimic action. The antiviral activity of these mimics can further be assessed by determining the viral titers in the presence or absence of these dsRNA molecules. Additionally, infection experiments in the presence of these mimics needs to be validated in mouse cells and in-vivo. The E1A expression experiment suggested that the infection is likely to be inhibited at the early stages of the infection. However, experiments can be planned (For example, viral transcriptome analysis upon mimic transfection) to further identify the specific steps in the infection programme of the cells, affected by these mimics. Result obtained from such studies can be compared with the predicted viral targets obtained from the bioinformatic analyses of miRNA-viral gene interactions. To test the functional relevance of type 1 IFNs induced by these mimics and to determine if the antiviral activity requires the type 1 IFN receptor in a feedback mode of action, infection assays can be performed in the mimic transfected IFN-receptor knock-out cells.

Summing up the major findings from this work, I, with the help of my co-workers found a novel way to block virus infections using double stranded, blunt ended and non-modified miRNA mimics of miR-29b-1* and miR-27b* in the cultured cells. With the capacity to activate antiviral innate immune response, these short dsRNA molecules could function as broad-spectrum antiviral agents. Compared to other non-specific dsRNA molecules like Poly I:C based drugs, the mimics used in this study seem to utilize specific sequence motifs to block the infection. The efficient delivery of these dsRNA molecu

-les into patient cells is challenging. And, also there is problem of toxicity, although this toxicity might be more pronounced in the tissue culture cancer cells (Bader et al. 2010). Thus, by increasing the potency and the stability and reducing the toxicity, these dsRNA mimics could be used to study the viral pathogenesis and can be used to develop novel antiviral drugs.

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Abbreviations

AGO	Argonaute
AIM2	Absent in melanoma 2
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
ASC	Apoptosis-associated speck-like protein
BDV	Borna disease virus
BLV	Bovine leukaemia virus
Bta	Bos taurus
CAR/CXADR	Coxsackievirus and adenovirus Receptor
CARDs	Caspase activation and recruitment domains
CCHFV	Crimean-congo haemorrhagic virus
cGAS	Cyclic GMP-AMP synthase
cGAMP	Cyclic guanine-adenine dinucleotide
CLRs	C-type lectin receptors
CML-BC	Chronic myelogenous leukaemia
CTL	Cytotoxic T cell
Cy3	Cyanine 3
CYPA	Cyclophillin A
DAI	DNA-dependent activator of IFN-regulatory factors
DAPI	4’,6-diamidino-2-phenylindole
DBP	DNA binding protein
DENV	Dengue virus
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dNTP	Deoxynucleoside triphosphate
dsRNA	Double stranded RNA
DTT	Dithiothreitol
DUBs	Deubiquitylating enzymes
EBOV	Ebola virus
EBV	Epstein-Barr virus
eIf	Eukaryotic translation initiation factor
ENCODE	Encyclopedia of DNA Elements
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
EV71	Enterovirus 71
FCS	Fetal calf serum
FDR	False discovery rate
FF-Luc	Firefly luciferase
FMDV	Foot and mouth disease virus
GO	Gene ontology
GFP	Green fluorescent protein
GS	Goat serum
GTP	Guanosine triphosphate
HA	Hemagglutinin
HAdV	Human adenovirus

HAV	Hepatitis A virus
HBsAg	HBV surface antigen
HBV	hepatitis B virus
HCMV	Human cytomegalovirus
HCOV	Human coronavirus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency virus
HPIV1	human parainfluenza virus type 1
HPV	Human papillomavirus
Hsa	Homo sapiens
HSV-1	Herpes simplex virus-1
HRV1A	Human rhinovirus
IAV	Influenza virus
IFI16	IFNγ inducible protein 16
IFIT2	interferon-induced protein with tetratricopeptide repeats
IFN	Interferon
IKK	IkB kinase
IL	Interleukins
ISGs	IFN-stimulated genes
ISRE	IFN-stimulated response elements
IRF	interferon response factor
ITGAV	Integrin alpha-v
ITRs	Inverted tandem repeats
JCV	Human JC polyomavirus
JEV	Japanese encephalitis virus
JNK	c-Jun N-terminal kinases
kbp	Kilobase pair
Klc	Kinesin light chain
KSHV	Kaposi's sarcoma associated herpes virus
Log_2FC	Logarithmic fold change
MAMs	Mitochondrial associated membranes
MAPK	mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signaling protein
MDA5	Melanoma differentiation-associated gene 5
MDV1	Marek's disease virus 1
MERS-CoV	Middle east respiratory syndrome coronavirus
MeV	Measles virus
MLP	Major late promoter
MHC	Major histocompatibility complex
MHV	Murine gamma herpesvirus
miRNA	MicroRNA
miRNP	Micro-ribonuclear protein complex
MVBS	multivesicular bodies
MX1	Myxovirus resistance 1
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NiV	Nipah virus
NK	Natural killer cells
NLRs	NOD like receptors

NPs	Nucleoproteins
NPC	Nuclear pore complex
NS1	Non-structural protein-1
nsp	Non-structural protein
NUP	Nuclear pore complex-filament protein
OAS	Oligoadenylate synthase
ORF	Open reading frame
OTU	Ovarian tumour
PACT	Protein kinase activator
PAMPs	Pathogen associated molecular patterns
PAR-CLIP	Photo- activatable ribonucleoside-enhanced cross-linking and immunoprecipitation
PBS	Phosphate buffered saline
PEDV	Porcine epidemic diarrhoea virus
PDCS	Plasmacytoid dendritic cells
PFA	Paraformaldehyde
piRNA	Piwi-associated small RNA
PKR	Protein kinase R
PLP	Papain-like protease
Poly(I:C)	Polyinosinic: polycytidylic acid
PMSF	Phenylmethylsulfonyl fluoride
PP	Protein phosphatase
PRRs	Pattern recognition receptors
pTP	Terminal protein precursor
PTMs	Post translational modifications
qRT-PCR	Quantitative real-time polymerase chain reaction
rAdV	Replication deficient adenovirus
RNAi	RNA-interference
RdRP	RNA-dependent RNA polymerase
REN-Luc	Renilla Luciferase
RGD	The tripeptide Arg-Gly-Asp
RIG-I	Retinoic acid-inducible gene-I
RIN	RNA integrity number
RLRs	Retinoic acid-inducible gene-I (RIG-I)-like receptors
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNF	Ring finger proteins
RSV	Respiratory syncytial virus
SARS-CoV	Severe acute respiratory syndrome-associated coronavirus
SDS	Sodium dodecyl sulfate
SFTSV	Severe fever with thrombocytopenia syndrome virus
sfRNA	Small flaviviral RNA
SFV	Semliki forest virus
siRNA	Short interfering RNAs
SR	Scavenger receptor
sstDNA	Strong-stop DNA
STAT	Signal Transducer and Activator of Transcription
STING	Stimulator of interferon genes

SuccEst	Succinimidyl ester
SV40	Simian virus 40
TBK1	TANK Binding Kinase 1
TBST	Tris buffered saline with Tween
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TP	Terminal protein
TRAF	TNF Receptor Associated factor
TRIM	Tripartite motif protein
TX-100	TritonX-100
Ub	Ubiquitin
UTR	Untranslated region
VA-RNAs	Viral associated RNA
VIRF	Viral interferon regulatory factor
VP	Viral protein
VSV	Vesicular stomatitis virus
WNV	West nile virus

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About Me

Molecular Life Science specialist with 6 years experience in managing multiple research projects in Genomics and Virology. I am currently involved in developing broad-spectrum antiviral drug. I have a strong liking for Statistics and Data analysis. I am a big data enthusiast with keen interest in applying advanced analytics for mining large Datasets. I aim to work in an Organization with a primary focus on Data Statistics and Analytics.

Career Objectives

To work in a challenging environment by demonstrating my skills and abilities to attain experience and knowledge in an organization offering scope for career growth and contribute to the professional development by producing best results.

Education

Since 2012 **PhD (Molecular Life Sciences)**, *(pursuing) Life Science Graduate School, University of Zurich*, Switzerland, *(Expected passing year-2016)*.

Thesis (Lab of Prof Urs Greber): Innate sensing of miRNA mimetics provides broad range anti-viral effects.

2010 - 2011 **Master of Science (Analytical Genomics)**, *University of Birmingham*, United Kingdom, *Grade-Distinction (180 credits achieved)*.

Master Thesis (Lab of Prof Zewei Luo): Statistical Modelling and analysis of Microarray based gene expression and evaluation of Single Nucleotide Polymorphisms.

2005 - 2009 **Bachelor of Technology (Biotechnology)**, *Padmashree Dr.D.Y.Patil University*, India, *Grade - First class (Distinction in the dissertation project)*.

Bachelor Thesis (Dr Ganapathy Subramanian, National chemical Laboratory, India): Exploring Structural Propensities on a Denatured Small Ubiquitin like Modifier (SUMO) protein by Ab-Initio Quantum Chemical calculation. .

Professional Experience

Feb 2012 - Present **Doctoral Research @ Life Science Graduate School, University of Zurich, Switzerland**, *(Prof - Urs Greber)*, (pursuing).

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Currently working on investigating the effect of small RNA molecules known as miRNAs on Viral Infections. Tested the toxicity of miRNA molecules on different cell lines and correlated it to sequence specific motifs

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General Tasks:

Design and conduct experiments.

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Generation of high throughput Experimental data .

Key Statistical and Computational Tasks:

Perform RNA-sequence analysis **(R/Bioconductor)**.

Gene Expression and Pathway Modelling **(R/Bioconductor, Metacore, String)**.

Generation and Statistical analysis of high throughput data **(R programming, MATLAB)**

High Throughput Imaging and Analysis **(MATLAB and Cell Profiler)**.

Perform sequence alignment of microRNAs with mRNA **(R Programming)**.

Identifying MicroRNA Targets **(Python)**.

Key Communication Tasks:

Communicating results to the scientific community via presentations and publications.

Teaching and supervising students (in academia) and training other members of staff.

Writing research proposals and apply for funding and grant.

Carry out peer reviews of written publications and presentations to validate theories and inform research by participating in Journal Clubs.

Dec 2010 - Sept 2011 **Post Graduate Research @ University of Birmingham, United Kingdom**, *(Prof Zewei Luo)*.

Topic: *in-silico* Genomics *(eQTL and Genome Wise Association Studies)*.

Carried out the Identification and detection of Expression Quantitative trait Loci and its role in explaining the variation in the gene expression phenotypes.

Key Analytical Tasks:

Statistical Modelling of Large Hapmap Genome Data **(R Package (haplo.stats), Plink)**.

evaluation of Single Nucleotide Polymorphism **(R Package (SNPassoc), Plink)**.

Construction of Genetic linkage maps **(JoinMap, CRAN-Onemap)**.

Locating the Quantitative Trait Loci **(QTL Catographer, R/QTL)**.

Genomic analysis for Single Nucleotide Polymorphisms **(JoinMap, CRAN-Onemap)**.

Identification of Population Stratification effects and Quantitative traits **(EIGENSTRAT, PLINK)**.

Analysis of Genome-wide association studies **(PLINK, R/qtl)**.

Jan 2010 - Aug 2010 **Research Assistant @ National Chemical Laboratory, India**, *(Dr. Ganapati Subramanian)*.

Topic: *ab-initio* Proteomics *(Protein folding and ab-initio NMR)*.

Perform *ab-initio* Calculation of Chemical shifts of the denatured proteins with main focus on Protein folding problem.

Key Computational Biology Tasks:

Perform *ab-initio* Nuclear Magnetic Resonance Spectroscopy **(Gaussian 03 and GaussView)**.

Predict the structural propensities in proteins **(DISSPred, CARA, Topspin)**.

Set up of Atomic Orbital calculations using quantum chemistry **(Gaussian 03)**.

Oct 2009 - Dec 2009 **Research Assistant @ Tata Institute of Fundamental Research, India**, *(Prof R.V. Hosur)*.

Topic: *ab-initio* Proteomics *(Molecular Modelling of Protein Dynamics and Simulations).*

Carried out the *in-silico* simulation of folding and unfolding states of Protein upon denaturation.

Key Computational Biology Tasks:

Carry out Protein-ligand docking **(GROMACS (unix based), NAMD and Arguslab Docking suite)**.

Perform Molecular dynamics simulations of proteins **(GROMACS (unix based))**.

Generation of Protein Structures **(Pymol, Rasmol and molmol)**.

Data Management and Statistical skills

Data Analysis	Analyze/organize/work with data on computers by Statistical programming, experience in applying machine learning methods.
Data Management	Make Data Management decisions and apply visualization strategies with **SAS** and **Python**.
Statistical Modelling	Explanatory and response variables, Fitting models to data, Statistical adjustment, geometry of model fitting and performing Correlations as a measure of alignment.
Exploratory data analysis	Make plots, discover patterns with visualizations, explore assumptions, Calculate statistical parameters using '**R**' and '**SAS**'.
Analytical abilities	Apply descriptive statistics, including measures of central tendency, dispersion and association; inferential statistics including probability theory, Normal distributions and statistical significance, OLS regression, interactions, factor analysis, regression for categorical and limited dependent variables.

Computing Expertise

Statistical computing	R, SAS, Python, MatLab/Octave, SPSS.
Programming and database development	C/C++, Shell, Perl, Unix, SQL LaTeX.
OS knowledge	Linux, OSX, Windows.
Concepts	Structured Programming, Design Pattern and Refactoring, Cloud Computing, Quality Assurance.

Certified Courses

Computational

- Best Practices in programming
- Computational Biology
- Matlab for Biologists
- Scientific Programming in Python
- Study design and data analysis using the statistical software R-1
- Programming in Biology I (using python)
- Data Management and Visualization

Microscopy

- Winterschool 2013-Practical course in Advanced Microscopy
- Basics of Light Microscopy

Specialized Courses

- Flow Cytometry
- Structural Biology
- Microarray Technologies
- Systems Biology Module

Transferable Skills
- Business Concept
- German for late arrivals
- Open Access: Improvement of Scientific publishing

Publications

- Innate sensing of miRNA mimetics provides broad range anti-viral effects (manuscript in progress)
- ab initio calculation of NMR Chemical Shifts in Denatured Proteins and Prediction of Secondary Structural Preferences (Submitted to the Journal of Bimolecular NMR.

Extra-Curricular Activities and Achievements

Mar 2015 Business startup idea selected by Commission for Technology and Innovation (CTI), Switzerland.

Feb 2013 and 2014 ASVZ Zurich Marathon finisher - Running Challenge, Zurich Switzerland.

Other Hiking, Reading, Cycling

References

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